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Molecular and physiological approaches towards the characterisation of glycerol transport in *Saccharomyces cerevisiae*

Universidade do Minho
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Como acontece com quase tudo na vida, não há obras feitas exclusivamente por uma só pessoa. Mesmo que seja algo feito por um só há, no mínimo, alguém que influenciou, seja por intervenção directa com críticas ou sugestões ou, simplesmente, por um sinal discreto de apoio, de estímulo. Muitas vezes não é preciso estar presente nem que se faça ouvir o seu apoio para se sentir que não estamos sós. E isso pode ser fundamental não só para a realização do trabalho mas também para o melhorar. Estes pequenos sinais do dia a dia contribuem para a abertura de espírito que está tantas vezes aprisionado na rotina diária. Assim se consegue espírito analítico para a avaliação dos resultados e para o levantamento de novas questões. Para todos, muitos dos quais sem se aperceberem do seu contributo, vai o meu mais profundo agradecimento.

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Contents

Sumário

A adaptação fisiológica de células de *Saccharomyces cerevisiae* a condições de stresse salino envolve a acumulação intracelular de glicerol como soluto compatível. A concentração citoplasmática de glicerol é regulada permitindo a manutenção do equilíbrio da actividade da água entre o compartimento celular e o meio externo. Em células cultivadas em meios contendo açúcares fermentescíveis, tal como na maior parte dos habitats naturais de levedura, o glicerol é produzido por redução do intermediário metabólico gliceraldeído 3-fosfato, com envolvimento de NADH, e posterior desfosforilação. Uma vez que o cofactor NADH é produzido na glicólise, um mecanismo disponível para a regeneração de NAD^+ em condições de anaerobiose é através da síntese de glicerol. Assim, a elevada produção de glicerol é uma consequência fisiológica da fermentação em *S. cerevisiae*. Este glicerol sintetisado é libertado para o meio, permitindo a sua síntese continuada e regulação permanente do potencial redox. O incremento da síntese de glicerol como resposta a stresse osmótico é mediada pelo aumento da expressão de *GPD1*, que codifica a enzima glicerol 3-fosfato desidrogenase, e do gene *GPP2* que codifica a enzima glicerol 3-fosfato fosfatase. A acumulação é também conseguida por retenção de glicerol através do decréscimo da permeabilidade da membrana citoplasmática. O canal de glicerol Fps1p está envolvido nesta modificação da permeabilidade por um mecanismo de abertura e fechamento do poro. Por outro lado, uma influência na composição lipídica da membrana citoplasmática tem sido atribuída ao gene *FPS1*, podendo levar à diminuição da permeabilidade ao glicerol.

Neste trabalho são apresentados resultados que apontam para a identificação dos genes que codificam os sistemas de transporte activo do glicerol. O gene *GUP1*, previamente clonado através da dificuldade em utilização de glicerol como fonte de carbono e energia do respectivo mutante, é demonstrado neste trabalho estar envolvido no transporte activo de glicerol. Apesar de em mutantes *gup1* ser detectada uma cinética de saturação no transporte de glicerol, apenas em mutantes *gup1gut1* a componente de saturação do transporte de glicerol é completamente suprimida. A interferência do primeiro passo do catabolismo de glicerol, catalisado pela enzima glicerol cinase codificada pelo gene *GUT1*, na determinação experimental de transporte de glicerol em células desreprimidas é coerente com estes resultados. Em células cultivadas em glucose, o transporte activo de glicerol só foi detectado em células incapazes de sintetisar glicerol (fundo genético *gpd1gpd2*) na presença de stresse salino e de pequenas quantidades de glicerol no meio. Em mutantes com mutações adicionais nos genes *GUP1* e *GUT1*, este transporte continua a ser detectado. Um gene homólogo, posteriormente designado *GUP2*, foi demonstrado estar envolvido neste transporte uma vez que só se deixou de detectar transporte no mutante *gpd1gup1gup2*.

O envolvimento do transporte activo de glicerol na resposta a stresse osmótico tem sido sugerido por resultados já publicados para os genes *GUP1* e *GUP2*. A análise de expressão por quantificação relativa de mRNA por RT-PCR foi feita para confirmação deste envolvimento. Os níveis de mRNA detectados não foram coerentes com os resultados dos ensaios de transporte activo de glicerol marcado radioactivamente, uma vez que foram relativamente constantes para *GUP1* e *GUP2*. A possibilidade de regulação negativa por parte do glicerol sobre o transporte foi excluída devido à falta de correlação entre os valores de concentração intracelular e os resultados de transporte activo de glicerol. A possibilidade de crescimento sob stresse salino severo de mutantes incapazes de sintetisar glicerol, aponta para a participação da via da dihidroxiacetona na

síntese de glicerol. Esta conclusão é também apoiada na detecção de quantidades significativas deste composto no interior destas células mutantes.

O mecanismo de transporte de glicerol pelo canal de glicerol Fps1p foi também investigado neste trabalho devido à incoerência de resultados, publicados previamente, de ensaios de transporte e o tipo de transporte aceite para este sistema. Uma vez que em mutantes *fps1* é detectada uma cinética de saturação para a entrada de glicerol apenas em células cultivadas em glucose e colhidas na transição de metabolismo fermentativo para respiratório, uma possível interferência nos ensaios de transporte por desrepressão parcial do gene *GUT1* foi postulada. Na pesquisa de um componente saturável na cinética de transporte de glicerol nas condições referidas, apenas em mutantes *gut1* não se detectou este componente. Assim, com base nestes resultados e em resultados publicados sobre decréscimo da difusão passiva em mutantes *fps1* e na baixa lipossolubilidade da molécula de glicerol, o mecanismo de difusão simples através do canal Fps1p, em oposição à difusão facilitada, é sugerido neste trabalho.

Neste trabalho, os mecanismos de transporte transmembranar de glicerol foram estudados e caracterizados. A clonagem dos genes que codificam os sistemas de transporte activo de glicerol foi obtida com o contributo deste trabalho na caracterização dos sistemas de transporte a nível fisiológico e a nível da expressão destes genes, incluindo o envolvimento na resposta a stresse osmótico. O mecanismo de transporte de glicerol pelo canal Fps1p foi elucidado e a difusão simples através da bicamada lipídica da membrana plasmática também foi investigada. Com os resultados obtidos neste trabalho foi possível melhorar o modelo de transporte de glicerol que inclui quatro situações fisiológicas: repressão catabólica, desrepressão, desrepressão parcial e stresse salino. A expressão a nível da transcrição dos genes *GUP1* e *GUP2* é constante nestas condições e o transporte activo de glicerol só é detectado em células desreprimidas e em células sujeitas a stresse salino desde que incapacitadas de sintesar glicerol. A difusão simples é maioritariamente mediada pelo canal Fps1p e, tal como demonstrado previamente, é abolida sob stresse salino, permitindo retenção intracelular do glicerol.

Abstract

Physiological adaptation to salt stress of *Saccharomyces cerevisiae* cells involves intracellular accumulation of the compatible solute glycerol. Regulation of cytosolic glycerol concentration allows maintenance of balanced water activity between the intracellular compartment and the external medium. When cells grow on substrates containing fermentable sugars, as in most common yeast natural habitats, glycerol is produced by NADH-dependent reduction of the glycolytic intermediate glyceraldehyde 3-phosphate and subsequent dephosphorylation. Since a NADH surplus is produced in cells growing under anaerobic conditions, a mechanism to regenerate NAD^+ is by glycerol synthesis. Therefore, high glycerol production is a physiologic consequence of anaerobic growth of *S. cerevisiae* cells. This synthesised glycerol is released to the external medium allowing continuous synthesis of this compound and permanent redox balance. Increased synthesis of glycerol in osmotic stress response is mediated by higher expression of *GPD1*, encoding the key enzyme of glycerol anabolism, glycerol 3-phosphate dehydrogenase, and of *GPP2*, encoding glycerol 3-phosphate phosphatase. Accumulation is also achieved by retention of glycerol through a decrease of plasma membrane permeability to glycerol. The glycerol facilitator Fps1p is involved in this permeability modification by a mechanism of opening and closure of the pore. In addition, interference in lipid composition of the plasma membrane, presumably leading to decreased glycerol permeability, has been attributed to *FPS1*.

In this work, strong evidence is presented for the identification of the genes coding the glycerol active uptake systems. The gene *GUP1*, previously cloned by the impairment to utilise glycerol as carbon and energy source in the correspondent disruption mutants, is demonstrated here to be involved in glycerol active uptake. Despite the fact that in single *gup1* mutants a saturable kinetics of glycerol transport was detected, this residual uptake was completelly abolished in the *gup1gut1* mutant. An interference of the first catabolic step of glycerol, catalysed by glycerol kinase encoded by *GUT1*, with experimental determinations of uptake in derepressed cells is consistent with these results. In glucose-grown cells, glycerol uptake is only detectable in mutants unable to synthesise glycerol (*gpd1gpd2* genetic background) grown under salt stress in the presence of extracellular glycerol. In addition, further disruptions of *gup1* and *gut1* did not abolish this uptake. A homologous gene, named *GUP2* thereafter, was implicated in the uptake under these conditions since only *gpd1gup1gup2* mutant cells did not present this uptake.

Evidence pointing to an involvement of active uptake for glycerol in osmotic stress response has been reported for *GUP1* and *GUP2*. Expression analysis by relative quantification of transcripts by relative quantitative RT-PCR was performed in order to further confirm this involvement. Transcript levels did not match with radiolabelled glycerol uptake assays, since nearly constant levels were detected for both *GUP1* and *GUP2*. The possibility of negative feedback regulation of uptake by glycerol was excluded because intracellular levels of this compound did not match with uptake data. Interestingly, *gpd1gpd2* mutant strain cells were able to grow under severe salt stress and accumulate significant amounts of glycerol. These results strongly suggest a role in glycerol synthesis by the alternative dihydroxyacetone pathway.

The mechanism of glycerol transport by the glycerol facilitator Fps1p was investigated due to inconsistent data concerning the generally accepted mechanism of facilitated diffusion and the glycerol uptake assays reported

before. Since *fps1* mutant cells still presented glycerol uptake according to a saturation kinetics in glucose-grown cells harvested in the diauxic shift, the glycerol facilitation mechanism was questioned and an interference by partial derepression of the glycerol kinase encoded by *GUT1* was demonstrated since only *gut1* mutants are devoid of this saturable component. Based in these data and in previously reported data concerning decrease of passive diffusion in *fps1* mutants together with the low lipossolubility of glycerol, a mechanism of channel for glycerol mediating simple diffusion is presented for Fps1p.

In this work, the mechanisms of transmembrane transport of glycerol were further studied and characterised. A contribution was made to clone the gene encoding the glycerol active uptake system and to the characterisation of this transport system, both at physiological and transcript expression levels, including involvement in osmotic stress response. The mechanism of Fps1p-dependent glycerol transport and simple diffusion through the lipid bilayer of the plasma membrane was investigated. Data obtained in this work allowed an improvement of the model for the transmembrane transport of glycerol including four different physiological conditions: under catabolite repression, derepression, partial derepression and salt stress. Expression at transcriptional level of *GUP1* and *GUP2* is constant in these conditions and active glycerol uptake is detected only in derepressed cells and under salt stress in cells impaired in glycerol synthesis. Simple diffusion is largely mediated by Fps1p and, as demonstrated before, is only abolished under salt stress, contributing to retention of glycerol inside the cell.

Chapter I

General Introduction

Introduction

Glycerol (1,2,3-propanetriol) is a poly-hydroxy alcohol with three carbon atoms, obtained, essentially, as by-product from yeast ethanol fermentation and from fabrication of soaps and detergents from oils and fats. At normal ambient conditions, glycerol is a highly viscous liquid with higher density than water, colourless, odourless, with sweet warm taste, and freely miscible with water. These properties make of glycerol a chemical with a wide use in industry, ranging, for instance, from food industry as solvent, humectant, and sweetener; in cosmetics industry as solvent, humectant, and emollient; in engines as anti-freezing agent; and in biotechnology as stabiliser of enzyme solutions. The metabolic process resulting in considerable production of glycerol is ethanol fermentation and is performed with high efficiency by the yeast *Saccharomyces cerevisiae*. Smoothness and consistency of natural products of yeast fermentation like wine and beer are valorised and are characteristics conferred by glycerol synthesised and exported by yeast cells.

In living beings, glycerol is found in its free form or, more commonly, chemically bonded by ester linkages to fatty acids (triglycerides) or to fatty acids and phosphate (phospholipids). The metabolic pathways of glycerol (Fig. I.1) are a branch at the upper part of glycolysis at the level of dihydroxyacetone phosphate. This suggests that changes in glycerol metabolism can have influence in glycolysis, pointing to a potential regulation role for glycerol metabolism. Moreover, the involvement of redox and kinase/phosphatase reactions is in accordance with this regulator role through participation in global redox balance and inorganic phosphate turnover.

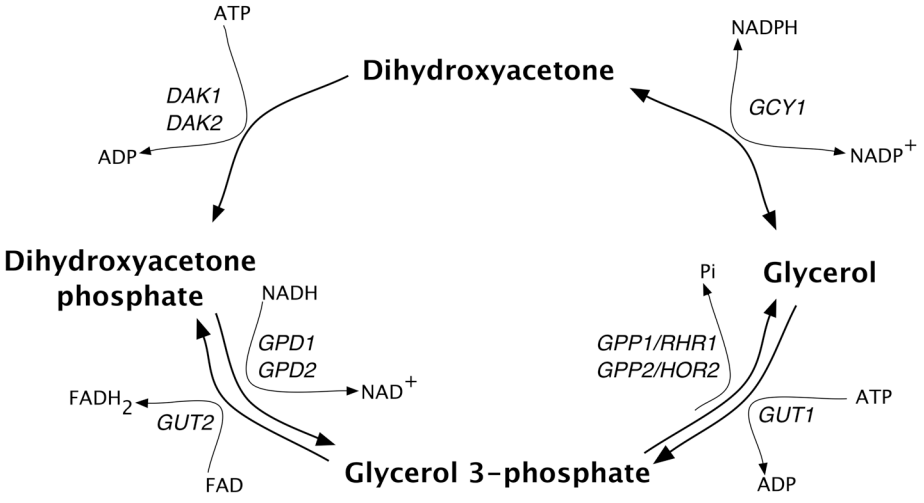

Figure I.1. Metabolic pathways of glycerol in *Saccharomyces cerevisiae*. In each metabolic step the gene(s) known to encode the enzyme, or different isoforms, catalysing the respective metabolic reaction are represented: Gpd1p and Gpd2p, glycerol 3-phosphate dehydrogenase; Gpp1p/Rhr2p and Gpp2p/Hor2p, glycerol 3-phosphate phosphatase; Gut1p, glycerol kinase; Gut2p, mitochondrial FAD-dependent glycerol 3-phosphate dehydrogenase; Dak1p and Dak2p, dihydroxyacetone kinase; Gcy1p, glycerol dehydrogenase.

Involvement of glycerol in yeast cell physiology

Glycerol as carbon and energy source

Glycerol catabolism

Glycerol catabolism in yeast cells involves transmembrane transport and two further enzymatic steps: phosphorylation catalysed by glycerol kinase (EC 2.7.1.30) and dehydrogenation catalysed by mitochondrial glycerol 3-phosphate dehydrogenase (EC 1.1.99.5). The remaining catabolic steps are included in gluconeogenesis, being involved, in opposition to other non-fermentable carbon sources, only one gluconeogenic-specific enzyme: fructose 1,6-bisphosphatase (EC 3.1.3.11). Uptake has been considered to be exclusively by simple diffusion (Gancedo *et al.,* 1968) due to the claimed lipid solubility of glycerol. Recently, however, mediated diffusion (Luyten *et al.,* 1995; Sutherland *et al.,* 1997) and active uptake (Lages and Lucas, 1997; Sutherland *et al.,* 1997) were detected. The correspondent genes encoding the glycerol channel for diffusion, *FPS1* (Luyten *et al.,* 1995), and the putative active transport protein, *GUP1* and *GUP2* (Holst *et al.,* 2000), were cloned and the respective mutants analysed. Regulation of expression and physiological functions of these genes have been investigated and are discussed in detail in chapters III, IV, and V.

Mutants defective in glycerol kinase and glycerol 3-phosphate dehydrogenase activities were isolated by random mutagenesis and selection for absence of growth with glycerol as sole carbon and energy source (Sprague and Cronan, 1977). The gene encoding glycerol kinase (EC 2.7.1.30), *GUT1*, was cloned (Pavlik *et al.,* 1993) and regulation of expression was investigated, being repressed by glucose and derepressed in non-fermentable carbon sources (Sprague and Cronan, 1977; Grauslund *et al.,* 1999) in a *ADR1*-dependent manner (Pavlik *et al.,* 1993). The expression of *GUT1* is regulated by carbon source with involvement of several activation/repression systems. Opi1p mediates repression and Adr1p, Ino2p, and Ino4p mediate activation of *GUT1* (Grauslund *et al.,* 1999). Adr1p is involved in activation of expression of genes repressed by glucose like *ADH2*, encoding catabolic alcohol dehydrogenase (EC 1.1.1.1) (Bemis and Denis, 1988), *ACS1* encoding acetyl-CoA synthetase (EC 6.2.1.1) (Kratzer and Schüller, 1997), and genes required for peroxisome proliferation (Simon *et al.,* 1991). For *ADH2* expression, binding of Adr1p to the promoter requires Snf1p, suggesting that Adr1p activation of transcription of all its target genes including *GUT1* is dependent of Snf1p (Young *et al*., 2002b). Ino2p, Ino4p, as activators and Opi1p, as repressor, are required for regulation of genes encoding phospholipid biosynthesis like inositol 1-phosphate synthase (EC 5.5.1.4) encoded by *INO1* (Hoshizaki *et al.,* 1990; Nikoloff *et al.,* 1992; White *et al.,* 1991).

Rønnow and Kielland-Brandt (1993) cloned *GUT2* by functional complementation of a previously isolated *gut2* mutant deficient in mitochondrial glycerol 3-phosphate dehydrogenase activity (Sprague and Cronan, 1977). The

enzyme encoded by this gene is a FAD-dependent glycerol 3-phosphate dehydrogenase (EC 1.1.99.5) located at the inner mitochondrial membrane by hydrophobic interactions (Janssen *et al.,* 2002) and catalyses the conversion of glycerol 3-phosphate to dihydroxyacetone phosphate. This compound is subsequently metabolised in glycolysis or gluconeogenesis. The expression of the FAD-dependent glycerol 3-phosphate dehydrogenase is, in accordance with expression of glycerol kinase, repressed by glucose and derepressed by non-fermentable carbon sources like ethanol, glycerol, and lactate (Sprague and Cronan, 1977). Similarly, for repression of expression the intervention of the negative regulator Opi1p is required but, for derepression, different transcription factors are required: the protein kinase Snf1p and the heteromeric complex Hap2/3/4/5 (Grauslund and Rønnow, 2000). The *SNF1* gene product plays an essential role in the glucose repression pathway by inactivating the Mig1p-Cyc8p-Tup1p repression complex, allowing derepression of several glucose-repressed genes like those involved in mitochondrial respiration, metabolism of alternative sugars, gluconeogenesis, and glyoxylate cycle (reviewed by Thevelein, 1994 and Gancedo, 1998). In addition, Snf1p might be involved in Adr1p-mediated transcription activation of *GUT1* (Young *et al*., 2002b), suggesting coordinated regulation of the kinase and dehydrogenase steps of glycerol catabolism. The Hap2/3/4/5 complex activates transcription when yeast cells utilise non-fermentable carbon sources for growth (reviewed by Gancedo, 1998 and Klein *et al.,* 1998). The genes activated by this complex are involved in mitochondrial electron transport chain, in heme biosynthesis (Keng and Guarente, 1987; Keng *et al.,* 1992), and Krebs cycle (Repetto and Tzagoloff, 1989; Repetto and Tzagoloff, 1990; Rosenkrantz *et al.,* 1994; Haviernik and Laquin, 1996; Liu and Butow, 1999). Other genes are controlled by the HAP complex, including genes involved in mitochondrial biogenesis (Dang *et al.,* 1994), tolerance to oxidative stress (Watt and Piper, 1997), and nitrogen metabolism (Georgakopoulos and Thireos, 1992; Dang *et al.,* 1996a). It is interesting to note the reported regulation of expression by the HAP complex of genes involved in nitrogen metabolism and of genes encoding enzymes of the Krebs cycle. In the regulated genes from nitrogen metabolism are included *ASN1* and *ASN2* putatively encoding asparagine synthetase (EC 6.3.5.4.) (Dang *et al.,* 1996b), *LPD1* encoding lipoamide dehydrogenase (EC 1.8.1.4) (Bowman *et al.,* 1992), and *GDH1* encoding NADP-dependent glutamate dehydrogenase (EC 1.4.1.4) (Dang *et al.,* 1996a; Riego *et al.,* 2002). In the case of the Krebs cycle, are included citrate synthase (EC 2.3.3.1) encoded by *CIT1* (Rosenkrantz *et al.,* 1994), aconitate hydratase (EC 4.2.1.3) encoded by *ACO1* (Haviernik and Laquin, 1996), dihydrolipoyl transsuccinylase (EC 1.2.4.2) encoded by *KGD1* (Repetto and Tzagoloff, 1990), and alpha-ketoglutarate dehydrogenase (EC 2.3.1.61) encoded by *KGD2* (Repetto and Tzagoloff, 1989). The possibility of a general control of Krebs cycle by the HAP complex contributes to explain this transcriptional regulator partition. In fact, the Krebs cycle is required for energy metabolism and for synthesis of precursors of amino acids (Dang *et al.,* 1996a).

Regulation of both glycerol kinase and mitochondrial FAD-dependent glycerol 3-phosphate dehydrogenase is still similar in terms of absence of catabolite inactivation. While activity of these enzymes increase, although with constant specific activities in control cultures without glucose, in cultures to which glucose is added, activities were still measured after 6 hours with a decrease of the specific activities (Sprague and Cronan, 1977). This is consistent with a regulation of glycerol kinase and mitochondrial glycerol 3-phosphate dehydrogenase activities by catabolite repression, as has been reported before

(Sprague and Cronan, 1977; Grauslund *et al*., 1999; Grauslund and Rønnow, 2000).

Triacylglycerol metabolism

Glycerol can be utilised directly for glycerophospholipid and triacylglycerol biosynthesis from its derivatives glycerol 3-phosphate and dihydroxyacetone phosphate (Fig. I.2). In the first step catalysed by glycerol 3-phosphate acyltransferase (EC 2.3.1.15), glycerol 3-phosphate is acylated with formation of 1-acyl glycerol 3-phosphate (lysophosphatidic acid) (Christiansen, 1978). Subsequently, lysophosphatidic acid is further acylated in a reaction catalysed by 1-acylglycerol 3-phosphate acyltransferase (EC 2.3.1.51), to form phosphatidic acid (Christiansen, 1978), which is the precursor of synthesis of all acylglycerol lipids. Alternatively, phosphatidic acid can be synthesised from dihydroxyacetone phosphate by acylation and subsequent reduction (Racenis *et al.,* 1992). The enzymes involved in this pathway are dihydroxyacetone phosphate acyltransferase (EC 2.3.1.42) for the first step and the NADPH-dependent 1-acyldihydroxyacetone phosphate reductase (EC 1.1.1.101) for the second one. Phosphatidic acid is subsequently converted, in a reaction catalysed by CDP-diacylglycerol synthase (E.C. 2.7.7.41), to CDP-diacylglycerol (Kelley and Carman, 1987), a precursor of glycerophospholipids like phosphatidylserine, phosphatidylcholine, phosphatidylethanolamine, and cardiolipin. On the other hand, phosphatidic acid can be converted to diacylglycerol, a precursor of triacylglycerol and phosphatidylethanolamine and phosphatidylcholine, in a dephosphorylation reaction catalysed by phosphatidate phosphatase (E.C. 3.1.3.4) (Hosaka and Yamashita, 1984). Hence, phosphatidic acid is a key intermediate in lipid metabolism: it is a general precursor of all acylglycerol lipids and it is at the branching point between synthesis of triacylglycerol and glycerophospholipids.

The genes encoding the enzymes of the metabolic routes leading to phosphatidic acid synthesis were already cloned and characterised. The open reading frame *YBL011w*, previously named *SCT1* for choline transporter suppressor, encodes the enzyme glycerol 3-phosphate acyltransferase involved in acylation of glycerol 3-phosphate. The open reading frame *YKR067w*, encoding a protein with sequence similarities to Ybl011wp, was demonstrated to be involved in this metabolic step as well, by analysis of the correspondent disruption mutant (Zheng and Zou, 2001). These open reading frames, *YBL011w* and *YKR067w*, were named *GAT2*(*SCT1*) and *GAT1*, respectively. The specificity for substrate of Gat1p and Gat2p is, according to biochemical analysis, the same for glycerol 3-phosphate and dihydroxyacetone phosphate (Athenstaedt *et al*., 1999; Zheng and Zou, 2001). Both glycerol 3-phosphate acyltransferase and dihydroxyacetone phosphate acyltransferase activities were detected in the lipid particle fraction and in the endoplasmic reticulum (Christiansen, 1978; Zinser *et al.,* 1991; Athenstaedt and Daum, 1997; Athenstaedt *et al.,* 1999). For lipid particles, these activities were attributed to Gat1p since none of these enzymatic activities were detected in this cellular fraction obtained from *gat1* mutants (Athenstaedt *et al.,* 1999). In the case of the endoplasmic reticulum, still some acyltransferase activity using glycerol 3-phosphate and dihydroxyacetone phosphate as substrates was detected in the microsomal fraction of the *slc1gat1* mutant, which suggests the existence of Gat2p in this organelle (Athenstaedt and Daum, 1997; Athenstaedt *et al.,* 1999).

Another organelle in which is detected acyltransferase activity is mitochondrion. Contrarily to lipid particles and endoplasmic reticulum, acyltransferase activity in the mitochondrion is preferentially devoted to acylation of dihydroxyacetone phosphate rather than glycerol 3-phosphate, suggesting the existence of a different dihydroxyacetone phosphate acyltransferase (Athenstaedt *et al.,* 1999). Other cellular fractions were demonstrated to contain only residual activity like nuclear membrane, plasma membrane, vacuoles, and peroxisomes (Zinser *et al*., 1991).

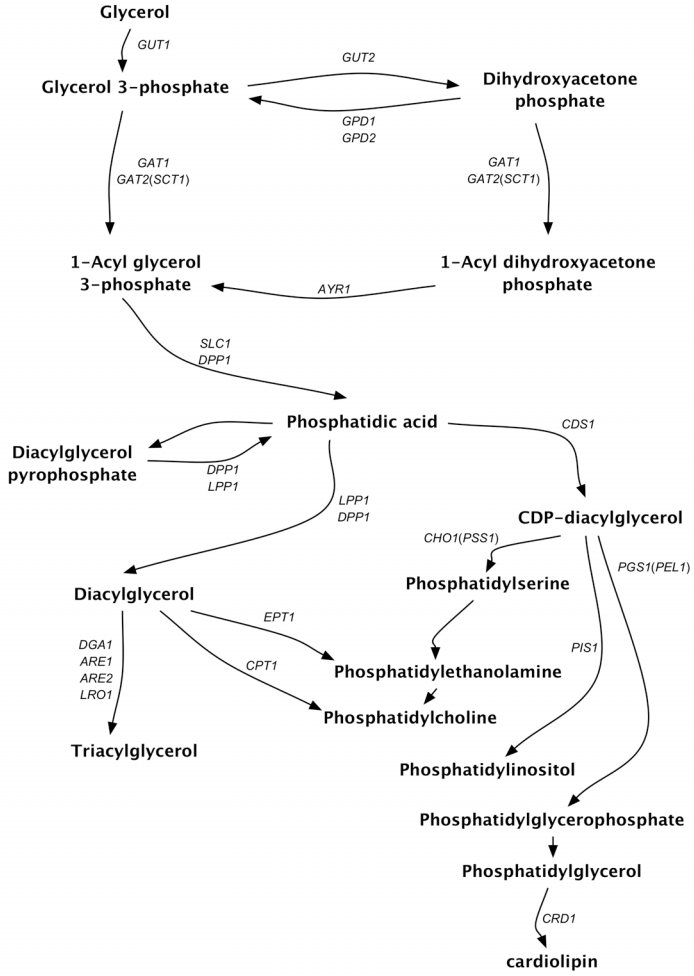

Figure I.2. Metabolic pathways of triacylglycerol and glycerophospholipids in *Saccharomyces cerevisiae*. The genes encoding enzymes with demonstrated *in vitro* catalytic activity are represented in each respective metabolic reaction. The enzymes are glycerol 3-phosphate acyltransferase, Gat1p and Gat2p(Sct1p); 1-acyldihydroxyacetone phosphate reductase, Ayr1p; 1-acyl glycerol 3-phosphate acyltransferase, Slc1p; diacylglycerol pyrophosphate phosphatase, Dpp1p; phosphatidate phosphatase, Lpp1p; acyl-coenzyme A:diacylglycerol acyltransferase, Dga1p; acylCoA:sterol acyltransferase, Are1p and Are2p; phospholipid:diacylglycerol acyltransferase, Lro1p; CDP-ethanolamine: 1,2-diacylglycerol ethanolaminephosphotransferase, Ept1p; cholinephosphate:CTP cytidyltransferase, Cpt1p; CDP-diacylglycerol synthase, Cds1p; phosphatidylserine synthase, Cho1p(Pss1p); phosphatidylinositol synthase, Pis1p; phosphatidylglycerophosphate synthase; Pgs1p(Pel1p); cardiolipin synthase, Crd1p.

The enzyme 1-acyldihydroxyacetone phosphate reductase is encoded by *AYR1* (Athenstaedt and Daum, 2000). Ayr1p was detected by Western blot analysis in lipid particles and in the microsomal fraction. These localisation studies were consistent with enzymatic analysis of subcellular fractions of wild-type cells. However, as *ayr1* mutants presented still considerable 1-acyldihydroxyacetone phosphate reductase activity in the microsomal fraction, an additional Ayr1p-like reductase might be present in the endoplasmic reticulum, presumably encoded by a different gene.

The gene encoding the enzyme involved in the subsequent step of acylation of 1-acyl glycerol 3-phosphate leading to phosphatidic acid, *SLC1*, was cloned by Nagiec and co-workers (1993) by amino acid sequence homology of the predicted translational products with the *Escherichia coli plsC* gene and by complementation of the growth defect of the *E. coli* mutant in this gene. Slc1p activity is detected in lipid particles and in the microsomal fraction. Interestingly, in *slc1gat1* mutant cells, a residual acyltransferase activity leading to phosphatidic acid synthesis is still detected in the microsomal fraction, which is sufficient to maintain the ability of growth (Athenstaedt and Daum, 1997). This clearly indicates that another acyltransferase using 1-acyl glycerol 3-phosphate as substrate is present in the endoplasmic reticulum.

The gene encoding phosphatidate phosphatase *LPP1* was cloned by sequence homology of part of the predicted product of the mammalian Mg^{++}-independent phosphatidate phosphatase and of the yeast diacylglycerol pyrophosphate phosphatase encoded by *DPP1* (Toke *et al*., 1998a; Toke *et al*., 1998b). Diacylglycerol pyrophosphate can be produced from phosphatidic acid by phosphorylation catalysed by phosphatidate kinase and converted back to phosphatidic acid in a reaction catalysed by diacylglycerol pyrophosphate phosphatase. Both Lpp1p and Dpp1p can utilise phosphatidic acid and diacylglycerol pyrophosphate as substrates, although with distinct specificities: while Lpp1p has greater specificity to phosphatidic acid, Dpp1p removes phosphate preferentially from diacylglycerol pyrophosphate. Mutant analysis revealed that Dpp1p is responsible for most of the cell phosphatase activity using as substrate phosphatidic acid, 1-acyl glycerol 3-phosphate, and diacylglycerol pyrophosphate (Toke *et al*., 1998a; Toke *et al*., 1998b). Biochemical analysis with the purified enzyme corroborated these observations in what concerns phosphatidic acid and diacylglycerol pyrophosphate utilisation as substrates by diacylglycerol pyrophosphate phosphatase (Wu *et al*., 1996). In addition, *lpp1* single deletion mutants lack an identifiable phenotype, while *lpp1dpp1* double mutants exhibit a marked decrease, although not total, in phosphatidate phosphatase and 1-acyl glycerol 3-phosphate phosphatase activities. However, diacylglycerol pyrophosphate phosphatase activity is completely abolished both in *dpp1* and in *lpp1dpp1* mutants. Therefore, the remaining phosphatase activity for phosphatidic acid and 1-acyl glycerol 3-phosphate should be the consequence of additional gene(s) involved in this metabolic pathway. The overlapping of substrate utilisation observed *in vitro* may not be the actual specificity of these enzymes *in vivo*, since cell compartmentation may impose the substrate(s) to be dephosphorylated by a given enzyme. According to Toke and co-workers (1998b), Lpp1p and Dpp1p activities were detected in total membrane fraction hence further investigation is needed to clarify this issue.

The final step in triacylglycerol synthesis from glycerol 3-phosphate and dihydroxyacetone phosphate involves the enzyme acyl-coenzyme

A:diacylglycerol acyltransferase (EC 2.3.1.20), which catalyses the acylation of diacylglycerol (Christiansen, 1978), and is encoded by *DGA1* (Sorger and Daum, 2002). Subcellular fraction analysis revealed a localisation of acyl-coenzyme A:diacylglycerol acyltransferase activity mainly in lipid particles with significant activity in the endoplasmic reticulum. The *dga1* deletion strain still presented considerable activity in the microsomal fraction, suggesting the existence of isoenzymes in this fraction. Recently, however, the acylCoA:sterol acyltransferases (EC 2.3.1.26) encoded by *ARE1* and *ARE2* (Yang *et al*., 1996; Yu *et al*., 1996) were reported to be involved in triacylglycerol biosynthesis as well (Sandager *et al*., 2000; Sandager *et al*., 2002; Oelkers *et al*., 2002), which could explain this phenotype of the *dga1* mutant. Additional systems for triacylglycerol biosynthesis were reported, based in acyl-CoA-independent acylation of diacylglycerol. The acyl donor can be a phospholipid in a reaction detected in the microsomal fraction and catalysed by phospholipid:diacylglycerol acyltransferase (EC 2.3.1.158) encoded by *LRO1* (Oelkers *et al*., 2000; Dahlqvist *et al*., 2000). On the other hand, acylation of diacylglycerol can be made with free fatty acids in a reaction independent of Dga1p and Lro1p and detected in lipid particles and in the microsomal fraction. The contribution of each system to total triacylglycerol is different, being the acyl-CoA- and the Lro1p-dependent systems the most important ones (Sorger and Daum, 2002).

Phospholipid metabolism

As described above, phosphatidic acid is at the branching point between triacylglycerol and phospholipid biosynthesis. However, phosphatidylethanolamine and phosphatidylcholine can be produced from diacylglycerol, the direct precursor of triacylglycerol. In these reactions included in the Kennedy pathway (Kennedy and Weiss, 1956), diacylglycerol is converted to phospholipid by reaction with CDP-ethanolamine and CDP-choline catalysed by CDP-ethanolamine: 1,2-diacylglycerol ethanolaminephosphotransferase (EC 2.7.8.1) encoded by *EPT1* (Hjelmstad and Bell, 1988) and cholinephosphate:CTP cytidyltransferase (EC 2.7.8.2) encoded by *CPT1* (Hjelmstad and Bell, 1987). Despite the importance of the Kennedy pathway, a central precursor of phospholipids is CDP-diacylglycerol (reviewed by Daum *et al*., 1998). CDP-diacylglycerol is produced in a reaction of phosphatidic acid with CTP catalysed by CDP-diacylglycerol synthase (EC 2.7.7.41) (Kelley and Carman, 1987) localised in the inner and outer mitochondrial membrane and in the endoplasmic reticulum (Kuchler *et al*., 1986). The gene encoding this enzyme, *CDS1*, was cloned by sequence homology of the predicted translational product with those of CDP-diacylglycerol synthase from *Drosophila melanogaster* and *Escherichia coli* and was demonstrated to be essential, due to spore germination and growth defects of the correspondent disruption mutants (Shen *et al*., 1996). Therefore, the metabolic routes involving directly phosphatidic acid and the branch for phospholipid biosynthesis are highly regulated. The shift from phospholipid to triacylglycerol synthesis has been reported to occur with inhibition of cell division (Graff and Lands, 1976) and the reverse was observed in early exponential growth phase (Taylor and Parks, 1979). These observations are in accordance with the generally accepted idea that triacylglycerols are produced as a storage form of energy and of fatty acids for membrane biosynthesis. Triacylglycerols are the main components of lipid particles, together with steryl esters (Leber *et al*., 1994), which are considered storage forms as well. Further evidence sustaining the requirement of phospholipid biosynthesis

for membrane formation was demonstrated by the involvement of the phospholipid biosynthesis positive regulator Ino2p (Loewy and Henry, 1984) in stimulated endoplasmic reticulum biogenesis (Block-Alper *et al*., 2002). The genes *INO2* and *INO4* were identified as being involved in increased expression of *INO1*, encoding the enzyme inositol 1-phosphate synthase, which catalyses the first step of inositol biosynthesis (Donahue and Henry, 1981; Loewy and Henry, 1984; Hirsch and Henry, 1986). On the other hand, Opi1p was demonstrated to be a negative regulator of transcription of *INO1* (Greenberg *et al*., 1982; Klig *et al*., 1985). The mechanism of inositol- and choline-mediated repression and depression of *INO1* involves formation of a heteromeric complex with Ino2p and Ino4p that binds to the upstream activation sequence at the promoter region of *INO1* (UAS_{INO}) under derepression conditions when inositol and choline are absent from the medium (Lopes and Henry, 1991; Nikoloff and Henry, 1994; Ambroziak and Henry, 1994; Schwank *et al*., 1995; Ashburner and Lopes, 1995a). The mechanism of *INO1* repression is still not entirely defined, although an interaction between the first element of regulatory input that would sense inositol and choline availability (Ashburner and Lopes, 1995b), Opi1p, and the pleiotropic repressor Sin3p has been shown to be crucial (Hudak *et al*., 1994; Wagner *et al*., 2001). This complex would bind the Ino2p/Ino4p heteromeric complex attached to UAS_{INO} (also mentioned as ICRE: inositol/choline–responsive element) and repression would occur through conformational changes leading to transcription blocking. This model is consistent with previous observations of the influence of gene dosage in UAS_{INO}-dependent transcriptional regulation. Over-expression of *INO2* suppresses the repression caused by phospholipid precursors (Schwank *et al*., 1997) and choline (Hosaka *et al*., 1994) and over-expression of *OPI1* causes auxotrophy for inositol (Wagner *et al*., 1999). Actually, the role of Opi1p has been mentioned as central in this regulation system, since derepression, according to this model, would be reached by inactivation of Opi1p (Ashburner and Lopes, 1995a) as was demonstrated in a deletion mutant at the promoter of *INO1* with constitutive expression independent of *INO2* and *INO4* (Swift and McGraw, 1995). In addition, the identification of UAS_{INO}-like elements at the promoters of *INO2* and *INO4* together with the observation that *INO2* expression is increased in response to inositol and choline suggests autoregulation of these genes. Therefore, this regulatory system would be cooperative through the simultaneous derepression of *INO2* and inactivation of *OPI1* to allow transcription (Ashburner and Lopes, 1995a). However, this autoregulation mechanism is not consistent with further work in which the promoter regions of *INO2* and *INO4* were reported to be not essential for transcriptional regulation (Schwank *et al*., 1997) and the expression of *INO4* was found constitutive regardless of the presence of an active *INO2* gene (Robinson and Lopes, 2000). Additional regulatory elements and promoter elements have been reported for regulation of expression of *INO2*. Transcription of the regulatory gene *INO2* is further controlled by an upstream essential sequence (UES) in the promoter which has been proposed as a divergent TATA element that would be responsible for the low level of transcription (Eiznhamer *et al*., 2001) and an upstream regulatory sequence (URS1) for binding of the so-called global negative regulatory gene product Ume6p (Jackson and Lopes, 1996). Interestingly, the effect of Ume6p is repression of *INO1* by recruiting of Sin3p and Rpd3p after binding of Ume6p to the URS1 of *INO1* promoter (Kadosh and Struhl, 1997) and derepression of *INO2* (Jackson and Lopes, 1996; Elkhaimi *et al*., 2000) through a mechanism not intirely defined which involves interaction with Sin3p and Opi1p (Kaadige and Lopes, 2003). Actually, Ume6p was found to act as a negative and positive

regulator of genes involved in phospholipid biosynthesis (Jackson and Lopes, 1996). *INO2* expression is regulated at translational level as well by an upstream open reading frame at the leader sequence of the mRNA that contributes to close control of translation (Eiznhamer *et al*., 2001). The presence of UAS_{INO} motifs in the promoter regions of many genes involved in phospholipid biosynthesis like *INO1* (Lopes and Henry, 1991), the phosphatidylserine synthase (EC 2.7.8.8) gene *PSS1*/*CHO1* (Kodaki *et al*., 1991; Bailis *et al*., 1992), the fatty acid synthase (EC 2.3.1.86) genes *FAS1* and *FAS2* (Schuller *et al*., 1992), the acetyl-CoA carboxylase (EC 6.4.1.2) gene *ACC1*/*FAS3* (Hasslacher *et al*., 1993), and the acetyl-CoA synthetase (EC 6.2.1.1) gene *ACS2* (Hiesinger *et al*., 1997), makes of Ino2p, Ino4p, and Opi1p the elements of the coordinated regulation of phospholipid biosynthesis.

The complexity of metabolic pathways in lipid metabolism parallels the mechanisms involved in its regulation. Besides UAS_{INO}-dependent regulation, evidence has been reported for additional regulatory mechanisms. An increase in phosphatidate phosphatase activity induced by inositol was reported through a mechanism independent of soluble effector molecules (Morlock *et al*., 1988) and biochemical control was observed in enriched extracts by inhibition of activity in the presence of CDP-diacylglycerol, diacylglycerol, and triacylglycerol (Lin and Carman, 1989). In addition, the inositol-mediated reduction of phosphatidylglycerophosphate synthase activity encoded by *PGS1* involved in cardiolipin synthesis was demonstrated to be independent of Ino2p, Ino4p, and Opi1p (Greenberg *et al*., 1988). Similarly to inositol, supplementation with unsaturated fatty acids can influence lipid metabolism, with interference in glycerol 3-phosphate acyl transferase activity (Casey *et al*., 1993). The enzyme CTP synthetase (EC 6.3.4.2) encoded by *URA7* (Ozier-Kalogeropoulos *et al*., 1991) and *URA8* (Ozier-Kalogeropoulos *et al*., 1994) genes is involved in production of CTP, a precursor of the intermediate in phospholipid biosynthesis CDP-diacylglycerol, and is regulated by CTP product inhibition (Ostrander *et al*., 1998). Furthermore, a $Glu^{161} \rightarrow Lys$ mutation in *URA7* caused decreased phospholipid/neutral lipids ratio concomitant to increased content of triacylglycerol, free fatty acids and ergosterol ester and a decrease in diacylglycerol. Hence, *URA7* seems to be important not only for regulation of the metabolic flux for synthesis of triacylglycerols or phospholipids but also for utilisation of the pathway for phospholipid biosynthesis since the above mentioned mutant displays an increase in the utilisation of the Kennedy pathway. Another regulatory mechanism in phospholipid biosynthesis pathway is the influence of the activity of CDP-diacylglycerol synthase encoded by *CDS1* over the downstream enzymes phosphatidylserine synthase encoded by *CHO1*/*PSS1* and phosphatidylinositol synthase encoded by *PIS1* and over inositol 1-phosphate synthase encoded by *INO1* (Shen and Dowhan, 1997). While phosphatidylserine synthase and inositol 1-phosphate synthase are increased by regulation at transcriptional level with decreased activity of CDP-diacylglycerol synthase, phosphatidylinositol synthase activity is regulated in opposite manner at post-transcriptional level. Expression of *PIS1* is in fact quite different from other phospholipid biosynthetic genes. Despite the presence of a UAS_{INO} element at the promoter, transcription is not influenced by inositol or choline, being reduced in cells grown in media containing glycerol as carbon source and increased with galactose as carbon source as compared to glucose-grown cells. The transcriptional regulatory protein Mcm1p mediates the repression of *PIS1* whereas the *SLN1* gene product, that modulates Mcm1p activity, mediates its induction (Anderson and Lopes, 1996). Carbon source-dependent regulation is

found as well in the *PGS1* gene encoding the mitochondrial enzyme phosphatidylglycerophosphate synthase involved in cardiolipin biosynthesis (Shen and Dowhan, 1998). In addition to this, mitochondrial function, inositol and CDP-diacylglycerol synthase activity regulate *PGS1* expression. The influence of inositol was similar to the one of choline and follows the general pattern of *INO2*, *INO4*, and *OPI1* dependence of phospholipid biosynthetic genes (Dzugasova *et al*., 1998). However, the expression of *CRD1*, encoding cardiolipin synthase, is independent of inositol and choline and, therefore, of Ino2p, Ino4p, and Opi1p regulatory elements (Jiang *et al*., 1999). According to the localisation of cardiolipin in mitochondrial membranes, *CRD1* expression is affected by factors influencing mitochondrial development and is essential for growth at elevated temperatures.

Sterol metabolism

Besides phospholipids, sterols are essential components of membranes in *S. cerevisiae*. An important function of sterols is membrane fluidity and permeability regulation being, this property demonstrated in yeast plasma and mitochondrial membranes (McLean-Bowen and Parks, 1981; McLean-Bowen and Parks, 1982). Biosynthesis of sterol involves over 20 biochemical reactions (reviewed by Daum *et al*., 1998). The first part of this process begins with acetyl-CoA and terminates with the formation of farnesyl pyrophosphate and is called the mavelonate or isoprenoid pathway. The distinction of this part of the pathway is due to the fact that the end product farnesyl pyrophosphate is the precursor for synthesis of several other important cellular substances like heme, quinones and dolichols and the participation in post-translational modifications of proteins like the *ras* proteins. Therefore, farnesyl pyrophosphate biosynthesis is not specific for sterol production. In the second part of the pathway, farnesyl pyrophosphate is converted into the fungal sterol ergosterol. Enzymes involved in late steps of ergosterol biosynthesis have been localised in the microsomal subcellular fraction (Nishino *et al*., 1981; Leber *et al*., 1998) and in lipid particles (Zinser *et al*., 1993; Leber *et al*., 1994; Leber *et al*., 1998). Ergosterol is present in highest amounts in plasma membranes and secretory vesicles, while esterified derivatives with fatty acids are present in lipid particles (Zinser *et al*., 1993). However, ergosterol esterification catalysed by two acylCoA:sterol acyltransferase isoenzymes encoded by *ARE1* and *ARE2* (Yang *et al*., 1996; Yu *et al*., 1996) occurs in endoplasmic reticulum (Zinser *et al*., 1993; Zweytick *et al*., 2000) and steryl ester hydrolysis in the plasma membrane and in secretory vesicles (Zinser *et al*., 1993). Evidence from mutant analysis shows that Are2p is mainly involved in production of ergosterol esters, while Are1p produces mainly esters of precursors of ergosterol, which suggests a role in storage of precursors that can be available for sudden need of biosynthesis of the final product ergosterol (Zweytick *et al*., 2000). Strikingly, *GUP1* and *GUP2*, involved in active uptake of glycerol (Holst *et al*., 2000), were included in the MBOAT (for membrane-bound *O*-acyl transferases) super-family together with *ARE1* and *ARE2* (Hofmann, 2000). The common features of this super-family are the existence of eight to ten transmembrane domains and a conserved region of the predicted translation products. Moreover, all members characterised for biochemical activity are involved in catalysis of transfer of organic acids to hydroxyl groups of molecules included in membranes. While this feature has been demonstrated for Are1p and Are2p (Yang *et al*., 1996; Yu *et al*., 1996), for Gup1p and Gup2p new functions are suggested, namely the involvement of

Gup1p in triacylglycerol and phospholipid biosynthesis as suggested by Oelkers and co-workers (2000).

The biosynthesis of ergosterol is a highly regulated process, requiring molecular oxygen (Jahnke and Klein, 1983) and heme as an enzymatic cofactor (Gollub *et al*., 1977). Heme and oxygen are also involved in transcriptional regulation of genes involved in ergosterol biosynthesis. Anaerobic conditions and mutants defective in heme biosynthesis present increased transcription of *ERG9*, a gene encoding squalene synthase (EC 2.5.1.21), involved in the first step of the ergosterol biosynthesis specific pathway (Kennedy *et al*., 1999). The genes *ARE1* and *ARE2* are also regulated by heme (Jensen-Pergakes *et al*., 2001) in a way that oxygen availability seems to be relevant (Valachovic *et al*., 2002). While *ARE1* transcription is increased in a heme-deficient strain under anaerobiosis, *ARE2* transcription is increased only in a strain able to synthesise heme under aerobiosis. Accordingly, the heme-activated protein Hap1p is required for optimal transcription of *ARE2* and the derepression of *ARE1* and *ARE2* in a *rox1* mutant strain confirms the oxygen-dependent regulation of transcription of these genes (Jensen-Pergakes *et al*., 2001). Moreover, metabolic intermediates in ergosterol biosynthesis are also involved in transcriptional regulation of several steps of this pathway as suggested by the increased transcriptional activity of *ERG9* in *erg3*, *erg7*, and *erg24* mutant strains (Kennedy *et al*., 1999). The same behaviour is observed for *ERG3* expression in *erg2*, *erg3*, *erg4*, *erg5*, *erg6*, and *HMG1*, encoding the enzyme of the mavelonate pathway hydroxymethylglutaryl-CoA reductase (EC 1.1.1.34), catalysing the ergosterol biosynthesis rate-limiting step (Arthington-Skaggs *et al*., 1996). Modulation of *ERG9* expression is mediated by several distinct transcription factors, rendering the first step of the second half of the ergosterol biosynthesis pathway highly regulated by diverse mechanisms. The transcription factor involved in oxidative stress response yAP-1 and the heme activator protein transcription factors, Hap1p and the Hap2/3/4p complex, are activators of expression. Contrarily, the phospholipid transcription factor complex Ino2/4p is a negative regulator (Kennedy *et al*., 1999). Another level of transcriptional regulation in *ERG9* by genes not related with the ergosterol biosynthetic pathway was reported by Kennedy and Bard (2001). The product of *TPO1* is a negative regulator and the product of *SLK19* is a positive regulator as demonstrated by promoter-reporter gene fusions. In addition, the transcriptional regulator Mot3p involved in vacuolar function and endocytosis represses as well *ERG9*, *ERG2*, and *ERG6* (Hongay *et al*., 2002). These observations suggest an involvement of ergosterol in endocytic membrane transport system. Ergosterol has been demonstrated to regulate transcription of genes involved in its own biosynthesis pathway, particularly the activation of *ERG3* expression in the absence of ergosterol (Smith *et al*., 1996) and the negative regulation in *are1are2* mutants with simultaneous decrease in sterol levels (Arthington-Skaggs *et al*., 1996).

Overview of regulation of lipid metabolism

Regulation of lipid metabolism is highly complex and involves mechanisms acting at different levels. This complexity suggests tight control on membrane composition and biogenesis, which is consistent with the plasma membrane function of physical cell integrity and as the first level of adaptation to environmental changes. One of the most intriguing features is the localisation in distinct cellular compartments of enzymes catalysing subsequent steps in a

given metabolic pathway. The example of the mitochondrion lacking Ayr1p but possessing dihydroxyacetone acyltransferase activity (Athenstaedt *et al.,* 1999; Athenstaedt and Daum, 2000) clearly suggests the involvement of intracellular trafficking of metabolic intermediates in order to synthesise phosphatidic acid. This could be a way to create several pools of important precursor molecules like phosphatidic acid allowing rapid synthesis of lipids to compensate localised depletion. This is consistent with the localisation of glycerol 3-phosphate acyltransferase and 1-acyldihydroxyacetone phosphate reductase activities simultaneously in lipid particles and in the microsomal fraction (Christiansen, 1978; Zinser *et al.,* 1991; Athenstaedt and Daum, 1997; Athenstaedt *et al.,* 1999; Athenstaedt and Daum, 2000). Another example is ergosterol esterification in the endoplasmic reticulum (Zinser *et al*., 1993; Zweytick *et al*., 2000) and the subsequent storage in lipid particles (Zinser *et al*., 1993). For incorporation of free ergosterol in plasma membrane, ester hydrolysis occurs in this organelle after mobilisation of steryl esters from lipid particles.

Redundancy is found in several metabolic steps and contributes to high complexity of lipid metabolism and to successful formation of required lipid compounds. This feature is revealed by several examples of mutants in a given gene involved in a metabolic step that are still viable. In many cases, these mutations do not even abolish the correspondent metabolic pathway. The enzymes encoded by *GAT1* and *GAT2* are active for both glycerol 3-phosphate and dihydroxyacetone phosphate (Athenstaedt *et al*., 1999; Zheng and Zou, 2001). In triacylglycerol biosynthesis other genes than *DGA1* are involved such as *ARE1* and *ARE2* (Sandager *et al*., 2000; Sandager *et al*., 2002; Oelkers *et al*., 2002). Another example is the low substrate specificity for phosphatase activity of Lpp1p and Dpp1p (Toke *et al*., 1998a; Toke *et al*., 1998b).

Regulation at transcription level is mediated by the positive regulatory factors Ino2p and Ino4p and the negative regulatory factor Opi1p, which are common to almost all genes involved in phospholipid biosynthesis (Hoshizaki *et al.,* 1990: Nikoloff *et al.,* 1992; White *et al.,* 1991). However, some exceptions are found that suggest physiological connection between phospholipid metabolism and carbon source utilisation such as the gene *PIS1*, in which are involved the transcription factor Mcm1p and a two-component regulator Sln1p (Anderson and Lopes, 1996), and the gene *PGS1* (Shen and Dowhan, 1998). In sterol metabolism transcriptional regulation is mediated essentially by the heme activator protein transcription factors Hap1p and the complex Hap2/3/4p (Kennedy *et al*., 1999). However, other transcription factors are found like yAP-1, involved in oxidative stress response; Ino2p and Ino4p; and Mot3p, involved in vacuolar function and endocytosis (Hongay *et al*., 2002). Transcription regulation is mediated as well by metabolic intermediates in both phospholipid and sterol metabolism and nutrient supplementation in culture media by mechanisms in which the mentioned transcription factors might be involved.

Involvement of glycerol in lipid metabolism

The involvement of glycerol in lipid metabolism has not been considered in most published research works. However, recently, new evidence is pointing to at least some connection between the metabolism of glycerol and lipids. The precursors for biosynthesis of the key metabolic intermediate for lipid metabolism, phosphatidic acid, are glycerol 3-phosphate and dihydroxyacetone

phosphate (Christiansen, 1978; Racenis *et al.,* 1992). In addition, in the regulation of transcription of *GUT1* and *GUT2*, the transcription factors Ino2p, Ino4p, Opi1p, and the complex Hap2/3/4p (Grauslund *et al*., 1999; Grauslund and Rønnow, 2000) are shared with many genes of phospholipid and sterol metabolism. All the transcription factors reported that regulate *GUT1* and *GUT2* expression lead to the suggestion of dual control of these genes of glycerol metabolism: Adr1p and the Hap2/3/4p complex would be involved in *GUT1* and *GUT2* expression, respectively, for growth on glycerol and Ino2p and Ino4p for *GUT1* expression in order to synthesise lipid components of membranes (Grauslund and Rønnow, 2000). Interestingly, repression of *INO2* expression by inositol and choline is less prominent when glycerol, rather than glucose, is being used as carbon and energy sources (Grauslund *et al*., 1999). An explanation for this is the seemingly higher phosphatidic acid pool when growth is performed with glycerol as carbon source that might cause activation of Ino2p and Ino4p transcription factors. For *GUT2* transcription, the involvement of factors that could be directly attributable to lipid metabolism regulation has not been reported so far however, the possibility of utilisation of glycerol 3-phosphate and dihydroxyacetone phosphate as phosphatidic acid biosynthesis precursors (Christiansen, 1978; Racenis *et al.,* 1992) is in accordance with a presumable absence of such control of *GUT2*.

Roles of glycerol in metabolic regulation

Redox balance

Glycerol is one of the most abundant by-products, during fermentation of glucose by *S. cerevisiae* cells. The formation of ethanol is a redox neutral process and in glycolysis, reducing equivalents are produced in the cytoplasm in the form of NADH. For reduction steps of anabolic reactions, NADPH is the main cofactor. Since yeasts lack transhydrogenase activity (Lagunas and Gancedo, 1973), the conversion of NADH in NADPH is not possible. The participation of glycolysis in anabolism, with involvement of the tricarboxylic acid cycle, causes an excess of NADH that, under aerobic conditions, is dissipated in the electron transport chain for ATP production. This transfer of reducing equivalents to the mitochondrion is achieved by the NADH dehydrogenase localised in the inner mitochondrial membrane, with the catalytic site facing outside (von Jagow and Klingenberg, 1970; de Vries and Marres, 1987), and by shuttle systems like the glycerol 3-phosphate shuttle (Larsson *et al*., 1998a). However, in anaerobiosis, the only way to regulate cytoplasmic redox balance is by production of glycerol (van Dijken and Scheffers, 1986; Björkqvist *et al*., 1997).

In aerobic conditions, the glycerol 3-phosphate shuttle has been reported to be relevant only at low growth rates, under starvation (Påhlman *et al*., 2001b; Påhlman *et al*., 2002), and in cooperation with the mitochondrial NADH dehydrogenase, although in lesser extent (Overkamp *et al*., 2000). So, the main system in cytosolic NADH oxidation is the mitochondrial NADH dehydrogenase encoded by *NDE1* and *NDE2* (Small and Mcalister-Henn, 1998; Luttik *et al*., 1998) homologous to *NDI1*, encoding the NADH dehydrogenase (EC 1.6.5.3) with the catalytic site facing the mitochondrial matrix (Marres *et al*., 1991). Nevertheless, the glycerol 3-phosphate shuttle is capable to sustain respiratory

growth at low dilution rates in chemostat cultures of *nde1nde2* mutants (Overkamp *et al*., 2000). However, alternative mechanisms for cytosolic NADH oxidation have been proposed based in metabolic flux analysis in *nde1nde2gut2* mutants: intramitochondrial oxidation of ethanol produced in fermentation and a redox shuttle involving ethanol and acetaldehyde that would link NADH cytosolic oxidation to internal NADH dehydrogenase (Luttik *et al*., 1998; Overkamp *et al*., 2000). Other redox shuttle mechanisms are plausible to exist in *S. cerevisiae* like a malate-oxaloacetate shuttle, a malate-aspartate shuttle and a malate-pyruvate shuttle but their *in vivo* activity remains to be demonstrated (Bakker *et al*., 2001).

The mechanism of action of the glycerol 3-phosphate shuttle involves the metabolic intermediates of glycerol metabolism dihydroxyacetone phosphate and glycerol 3-phosphate. These two compounds can cross freely the outer mitochondrial membrane. After reduction of dihydroxyacetone phosphate in a reaction catalysed by cytoplasmic glycerol 3-phosphate dehydrogenase (EC 1.1.1.8), the product glycerol 3-phosphate is oxidised to dihydroxyacetone phosphate by the mitochondrial FAD-dependent glycerol 3-phosphate dehydrogenase and returns to the cytoplasm (Fig. I.1). The consequence of this cycle is the conversion of reduction potential in the form of cytoplasmic NADH into mitochondrial $FADH_2$. The genes involved in the metabolic steps of this shuttle *GPD1* and *GUT2* were cloned and characterised (Rønnow and Kielland-Brandt, 1993; Larsson, *et al*., 1993; Albertyn *et al*., 1994b; Grauslund and Rønnow, 2000). For the dihydroxyacetone phosphate reduction step, two genes, *GPD1* and *GPD2*, encoding glycerol 3-phosphate dehydrogenase isoenzymes were cloned (Larsson, *et al*., 1993; Albertyn *et al*., 1994b; Eriksson *et al*., 1995). Although expression of *GPD2* is increased in anoxic conditions while *GPD1* expression is altered with osmotic stress (Ansell *et al*., 1997), under aerobic conditions only Gpd1p is committed to dihydroxyacetone phosphate reduction to glycerol 3-phosphate, since glycerol formation still occurs when *GPD2* is deleted, and not *GPD1,* in a *gut2* genetic background (Larsson *et al*., 1998a). Microaerobic conditions created with low oxygen concentrations in cells fermenting glucose have been reported to allow simultaneous respiration (Weusthius *et al*., 1994) and, presumably, oxidation of excess of NADH. Analysis of expression pattern of cells cultivated in continuous cultures under these conditions corroborated the role of glycerol production involvement for *GPD1* in the absence of osmotic stress (Costenoble *et al*., 2000). The gene encoding the mitochondrial FAD-dependent glycerol 3-phosphate dehydrogenase, *GUT2*, is under transcriptional regulation of Snf1p and the Hap2/3/4/5 protein complex involved in transcriptional activation of genes participating in catabolism of non-fermentable carbon sources (Grauslund and Rønnow, 2000), which is consistent with its role in glycerol 3-phosphate shuttle during growth on ethanol as carbon and energy source (Larsson *et al*., 1998a).

Under anaerobic conditions, only *GPD2* is involved in regulation of cytoplasmic NADH, since *gpd2* mutants exhibit a growth inhibition phenotype in anaerobiosis with concomitant intracellular accumulation of NADH. Consistently, this growth defect is relieved by addition of the NADH oxidizer acetaldehyde to the culture (Ansell *et al*., 1997). Therefore, the enzyme encoded by *GPD2* is involved in redox adjustments under anaerobic conditions, contributing to NADH oxidation by increasing production of glycerol (Björkqvist *et al*., 1997; Ansell *et al*., 1997; Larsson *et al*., 1998a; Nissen *et al*., 2000). Accordingly, under microaerobic conditions with high demand for NADH reoxidation (very low oxygen concentration), *GPD2* was shown to be strongly induced (Costenoble *et*

al., 2000). The involvement of *GPD2* in anaerobiosis seems to be in close relation with the mitochondrial function since expression is independent of *ROX1* and *ROX3* genes (Ansell *et al*., 1997) encoding transcription factors for hypoxic and global stress response gene regulation (Deckert *et al*., 1995a; Deckert *et al*., 1995b; Evangelista *et al*., 1996). In addition, iron starvation causes induction of *GPD2* expression with increased glycerol production (Ansell and Adler, 1999) due to depletion in iron-requiring heme proteins of the respiratory chain. Hence, iron-starvation conditions would be physiologically equivalent to anaerobiosis.

For the glycerol 3-phosphate dephosphorylation step of glycerol biosynthesis, two isoenzymes, encoded by *GPP1*/*RHR2* and *GPP2*/*HOR2*, have been identified and purified (Norbeck *et al*., 1996). Similarly to genes of the previous metabolic step, *GPP1*/*RHR2* and *GPP2*/*HOR2* are differently regulated at transcriptional level (Norbeck *et al*., 1996; Påhlman *et al*., 2001a). Whereas *GPP2*/*HOR2* expression is increased by osmotic stress, *GPP1*/*RHR2* expression is induced by anaerobiosis and microaerobic conditions at high demand for NADH reoxidation (Costenoble *et al*., 2000). Interestingly, compensation of glycerol 3-phosphate phosphatase activity by *GPP1*/*RHR2* under osmotic stress occurs, since *gpp2*/*hor2* mutants are not affected in osmotic sensitivity. However, *GPP2*/*HOR2* does not compensate for absence of phosphatase activity in *gpp1/rhr2* mutants for growth under anaerobiosis (Påhlman *et al*., 2001a). This characteristic of Gpp1p/Rhr2p and the co-regulation of expression of *GPP1*/*RHR2* and *GPD1* under microaerobic conditions (Costenoble *et al*., 2000) suggest physiologic cooperation and regulatory interaction between these two genes. Interestingly, compensation of lack of Gpd2p activity by *GPD1* is possible as well, since *GPD1* transcription is increased in the *gpd2* mutant (Valadi *et al*., 1998). In addition, since the expression pattern of *GPD2* is altered in *gpd1* mutants under these conditions, the regulatory interaction of *GPD1* might be extended to *GPD2* (Costenoble *et al*., 2000). In the so-called dihydroxyacetone pathway, expression of the genes *GCY1* and *DAK1* is insensitive to oxygen however, in *gpd2* mutants *GCY1* is repressed and *DAK1* induced with very low oxygen concentration (Costenoble *et al*., 2000). Nevertheless, expression of *GCY1* has been reported to be under repression in anaerobic conditions (ter Linde *et al*., 1999). Therefore, the genes *GCY1*, putatively encoding *S. cerevisiae* glycerol dehydrogenase (Hur and Wilson, 2000; Hur and Wilson, 2001) with high predicted amino acid sequence homology with the enzyme of *Aspergillus niger* (Norbeck and Blomberg, 1997) and *DAK1* encoding dihydroxyacetone kinase (Molin *et al*., 2003), could hence participate in redox metabolism. The cofactor of Gcy1p is NADPH (Hur and Wilson, 2000; Hur and Wilson, 2001) so, the dihydroxyacetone pathway could provide, together with the glycerol 3-phosphate pathway, a transhydrogenase system modulating the cytosolic levels of NADH and NADPH (Costenoble *et al*., 2000).

One consequence of redox balance adjusted by glycerol production in anaerobic metabolism is the coupling between the metabolisms of nitrogen and glycerol. Glycerol yield is dependent on the type of nitrogen source available in a given culture in glucose-fermenting cells. Higher yields are achieved with ammonium rather than amino acids as nitrogen source (Albers *et al*., 1996). The need for synthesis of amino acids contributes to higher production of cytoplasmic reduction equivalents in the form of NADH. Therefore, increased glycerol production assures maintenance of redox balance under these conditions. Actually, when a mixture of amino acids is used as nitrogen source the lowest yield in glycerol production is achieved (Albers *et al*., 1996), meaning the

minimum intervention of metabolic pathways for amino acid synthesis. This is in accordance with higher ethanol yields and lower glycerol yields observed in chemostat cultures during nitrogen limitation (Lidén *et al*., 1995). Alcohol dehydrogenase might be implicated in this coupling since its activity is lower, and glycerol production higher, in cells grown in media with inorganic nitrogen rather than amino acids as nitrogen source (Omori *et al*., 1995). The type of influence of amino acid anabolism on alcohol dehydrogenase activity remains to be elucidated. Hence, the increased glycerol production as a redox sink when nitrogen is supplied in the inorganic form could be the consequence of the excess of reduction equivalents produced in the amino acid biosynthesis and the low NADH oxidation by alcohol dehydrogenase.

Role in inorganic phosphate turnover

The importance of proper inorganic phosphate levels for glycolysis has been taken in consideration from analysis of mutants affected in regulation of glycolysis. The mutant affected in *TPS1* (also known as *BYP1*, *CIF1*, *FDP1*, *GGS1*, *GLC6*, and *TSS1*) encoding the 56 kDa subunit of the trehalose 6-phosphate synthase/phosphatase complex (Bell *et al*., 1992; Vuorio *et al*., 1993), besides the incapacity to accumulate trehalose (Charlab *et al*., 1985), displays a complex phenotype including inability of growth on glucose and other fermentable carbon sources with accumulation of glycolytic intermediate sugar phosphates like fructose 1,6-bisphosphate and deficiency in inactivation of fructose 1,6-bisphosphatase (EC 3.1.3.11) by glucose (van de Poll *et al*., 1974). In one of the models proposed to explain this phenotype, Tps1p would act as regulator of glucose influx into glycolysis by utilising glucose for synthesis of the carbohydrate storage disaccharide trehalose (Thevelein and Hohmann, 1995). Under normal conditions, trehalose synthesis would release inorganic phosphate that is required for the glycolytic reaction catalysed by glyceraldehyde 3-phosphate dehydrogenase (EC 1.2.1.12). In *tps1* mutants, regeneration of inorganic phosphate is not possible, resulting in accumulation of fructose 1,6-bisphosphate and glyceraldehyde 3-phosphate, which is the substrate of glyceraldehyde 3-phosphate dehydrogenase. Other co-factors are required for this glycolytic enzyme that could contribute for the lack of activity when depleted: ADP and NAD^+. However, the normal levels of ADP and the NAD^+/NADH ratio reported (Hohmann *et al*., 1993; van Aelst *et al*., 1991) are not consistent with such possibility.

Strong evidence for the inorganic phosphate depletion model came from suppression of the growth defect on glucose in the *tps1* mutant by the gene *FPS1* (van Aelst *et al*., 1991) and the gene *GPD1* (Luyten *et al*., 1995). The additional observation that over-expression of *FPS1* increases glycerol synthesis strongly supports the mechanism of suppression of the growth defect of the *tps1* mutant simply by increased glycerol production. This way, the glycolytic intermediates sugar phosphates accumulated would be degraded for glycerol production with concomitant release of inorganic phosphate that would allow resume of activity of glyceraldehyde 3-phosphate dehydrogenase. The role as glycerol channel mainly involved in glycerol efflux has been proposed based in these results, which is in accordance with the mechanism of inorganic phosphate level restoration by avoiding intracellular accumulation of glycerol (Luyten *et al*., 1995). Remarkably, the suppression effect by *FPS1* does not affect the other phenotypes observed in the *tps1* mutant such as the incapacity to accumulate

trehalose and the deficiency in inactivation of fructose 1,6-bisphosphatase in cells growing on glucose, which further supports this model. The fact that the involvement of glycerol metabolism in inorganic phosphate regulation is observed only in mutant strains like *tps1* raises the question of its importance and relevance in this regulation in wild type strains fully competent to synthesise trehalose. Nevertheless, it is likely that, at least, some degree of cooperation for inorganic phosphate turnover might occur between the metabolic pathways of trehalose and glycerol.

Roles of glycerol in stress responses

Role in osmotic stress response

When *S. cerevisiae* cells face osmotic shock, sudden cell shrinkage occurs which is proportional to the osmolarity of the medium. During the adaptation period, before growth resumes, cellular volume is partially restored with simultaneous increase of glycerol 3-phosphate dehydrogenase activity, increase in glycerol production and subsequent intracellular accumulation (Blomberg and Adler, 1989; André *et al*., 1991; Albertyn *et al*., 1994a). Recovery of cell volume is made by counterbalance of internal and external osmotic pressures due to intracellular accumulation of glycerol in an active process dependent on *de novo* protein synthesis (Blomberg and Adler, 1989; Albertyn *et al*., 1994a). This way, water can be retained in the cell ensuring maintenance of metabolic reactions. Glycerol is the only osmoregulatory solute detected in *S. cerevisiae* (Reed *et al*., 1987), and constitutes an example of a compatible solute since, besides its function as osmolyte, high intracellular concentrations are not deleterious to the cell (Brown and Simpson, 1972).

Intracellular accumulation of glycerol can be achieved by increased production and simultaneous retention as has been reported to occur in *S. cerevisiae* (Blomberg and Adler, 1989). The key enzyme in glycerol production in osmotic stress response is the isoform of glycerol 3-phosphate dehydrogenase encoded by *GPD1* (Larsson *et al*., 1993; Albertyn *et al*., 1994b). Under osmotic stress, expression of *GPD1* is increased by induced transcription, as higher levels of *GPD1* mRNA are detected by Northern analysis and microarrays (Albertyn *et al*., 1994b; Ansell *et al*., 1997; Rep *et al*., 2000; Gasch *et al*., 2000; Causton *et al*., 2001; Yale and Bohnert, 2001). Levels of Gpd1p are increased as well (Varela *et al*., 1992; Norbeck and Blomberg, 1997), corresponding to one of the most responsive genes to osmotic stress. Other gene with increased expression induced by osmotic stress is *GPP2*/*HOR2* measured as enzymatic activity of glycerol 3-phosphate phosphatase (Norbeck *et al*., 1996) which is the result of increased level of transcription (Rep *et al*., 2000; Gasch *et al*., 2000; Causton *et al*., 2001; Påhlman *et al*., 2001a; Yale and Bohnert, 2001) and protein synthesis (Norbeck and Blomberg, 1997). Unlike the *GPD1* and *GPD2* genes encoding isoforms of the same enzyme with involvement in considerable distinct physiologic mechanisms, *GPP1*/*RHR2* and *GPP2*/*HOR2* can substitute each other in osmotic stress response as evidenced by retention of osmoresistance of mutants affected in each of these genes (Påhlman *et al*., 2001a) and the increased levels of expression of both *GPP1*/*RHR2* and *GPP2*/*HOR2* under these conditions (Norbeck and Blomberg, 1997; Rep *et al*.,

2000; Påhlman *et al*., 2001a; Yale and Bohnert, 2001). Global approaches allowed the detection of unexpected osmotic-responsive genes like those involved in glycerol catabolism by the dihydroxyacetone pathway: *GCY1*, putatively encoding an aldo-keto reductase glycerol dehydrogenase (Hur and Wilson, 2000; Hur and Wilson, 2001) and *DAK1*, encoding dihydroxyacetone kinase (Molin *et al*., 2003). The increased expression of these genes was demonstrated at mRNA and protein levels (Norbeck and Blomberg, 1997; Rep *et al*., 2000; Causton *et al*., 2001; Yale and Bohnert, 2001). Under these conditions, the effect of Gcy1p and Dak1p is apparently to decrease intracellular glycerol levels. However, the level of induction of expression reported for these genes is much lower than those of *GPD1* and *GPP2*/*HOR2* with correlation of the specific enzymatic activities measured of the correspondent gene products (Albertyn *et al*., 1994b; Norbeck and Blomberg, 1997). Therefore, only a small portion of glycerol produced is catabolised, resulting in a net effect of overproduction. Nevertheless, a small portion of glycerol is being produced and dissimilated in a cycle that was proposed to provide a safety valve for ATP consumption (Blomberg, 2000). When cells face osmotic shock, growth is arrested or decreased resulting in less demand for ATP in anabolic reactions. Because the upper part of glycolysis requires ATP, in these conditions glycolytic flux is stimulated and the production of more ATP at the lower part of glycolysis will contribute to even higher stimulation. This condition of imbalance between the upper and lower parts of glycolysis has been designated as substrate-accelerated death (Teusink *et al*., 1998) and the activation of ATP-consuming futile cycles like glycerol and trehalose turnover would alleviate this imbalance (Blomberg, 2000).

Early reports on osmotic stress response of *S. cerevisiae* have mentioned glycerol retention contributing to intracellular accumulation through the activity of a hypothetical glycerol transport mechanism (Albertyn *et al*., 1994b; Luyten *et al*., 1995) and decrease permeability in plasma membrane (Blomberg and Adler, 1989). An active transport mechanism for glycerol was identified by radiolabelled glycerol uptake experiments, apparently without connection with osmoregulation (Lages and Lucas, 1997; Sutherland *et al*., 1997). However, cloning of the gene encoding the transport protein, *GUP1*, was based in decreased osmotolerance of mutants generated randomly with transposons in a mutant strain, *gpd1gpd2*, unable to synthesise glycerol (Holst *et al*., 2000). A second gene, *GUP2*, was identified by sequence homology with *GUP1* by these authors, which encodes a second putative glycerol transport protein. Transcription analysis of *GUP1* and *GUP2* revealed different regulation of each gene with increased transcriptional activity of *GUP2* in the strain with impaired glycerol synthesis under salt stress and with correlation with high glycerol uptake activity (Holst *et al*., 2000; Chapters III and IV). Therefore, evidence has been obtained pointing to some connection between glycerol active uptake and osmotic stress response. Moreover, a transport system for glycerol by facilitated diffusion, encoded by *FPS1*, has been identified and characterised and the correspondent gene cloned (van Aelst *et al*., 1991; Luyten *et al*., 1995; Sutherland *et al*., 1997). The mechanism of transport of Fps1p is still a matter of research with the recent proposal of a mechanism of simple diffusion through the Fps1p channel (see Chapter V; Oliveira *et al*., 2003). Independently of the transport mechanism, the involvement of Fps1p in osmotic stress response is made through the control of intracellular levels of glycerol by regulating its efflux. The long N-terminal domain of the protein was demonstrated to be involved in this process of opening/closing of the channel, which is in accordance with the hypo-osmotic shock sensitivity of

fps1 mutant strains (Tamás *et al*., 1999). Recently, a short conserved region in this domain was identified to be responsible for regulation of glycerol transport through Fps1p, contributing to its retention under hyper-osmotic stress (Tamás *et al*., 2003). Interestingly, deletion of *FPS1* has effects in other cellular processes, some of which are not directly involved in osmotic stress. The proportion of glycolipids and phospholipids in plasma membrane composition is affected by *FPS1*, which might explain the lower glycerol diffusion in *fps1* mutants and in accordance with a role in glycerol intracellular retention (Sutherland *et al*., 1997). Another effect caused by *FPS1* deletion is cell fusion deficiency (Philips and Herskowitz, 1997). This phenotype was explained with the need for proper osmotic balance in fusion during mating due to the process of local degradation and remodelling of the cell wall. The molecular mechanism proposed for this effect is the higher glycerol content of *fps1* mutant cells that would activate the Pkc1p pathway, which inhibits cell fusion. However, this effect and the reported influence of Fps1p in glycerol production (Luyten *et al*., 1995; Tamás *et al*., 1999; Remize *et al*., 2001) are not a direct influence of Fps1p, but an indirect consequence of altered glycerol content. A remarkable example of an effect of *FPS1* deletion that does not involve directly glycerol is the resistance to the trivalent metalloids arsenite and antimonite by a mechanism independent of the intracellular glycerol (Wysocki *et al*., 2001). These observations and the hypersensitivity to these metalloids of cells expressing a truncated version of Fps1p with unregulated open channel provide support to the proposed function of Fps1p directly in transport of arsenite and antimonite.

Substantial progress has been obtained on the understanding of osmotic stress response by yeast cells and in particular the role of glycerol in this process. However, it has been reported that osmosensitive mutant strains generated randomly were mainly affected in glycerol content and in glycerol 3-phosphate dehydrogenase activity being, some of them not affected in genes encoding enzymes directly involved in glycerol production (Brüning *et al*., 1998). It is remarkable that in this work, although selection of osmosensitive mutants has not been based in the inability for glycerol production, most of the mutants obtained were affected in this process, which is indicative of the importance of glycerol in osmotic stress response. On the other hand, the observation that mutations in genes not directly involved in glycerol production cause decreased content in glycerol and glycerol 3-phospahte dehydrogenase activity points to intracellular accumulation of glycerol as an integrated response to osmotic stress. In this respect, the possibility of high probability of mutation in genes encoding elements of signal transduction pathways is in agreement with this observation.

Role in oxidative stress protection

Oxidative stress occurs when high concentrations of redox-active oxygen intermediates are achieved intracellularly as a consequence of several metabolic reactions such as incomplete reduction of oxygen to water in respiration, ß-oxidation of fatty acids or by influence of various stress agents: radiation, metals, and drugs (reviewed by Ames *et al*., 1993). Examples of these redox-active oxygen intermediates are the superoxide radical, hydrogen peroxide, and the hydroxyl radical and are highly reactive molecules that can damage proteins, lipids, and DNA. A proteome-wide approach by two-dimensional gel electrophoresis of total proteins of cells untreated and treated with hydrogen

peroxide revealed marked alterations in levels of enzymes of glycerol metabolism and global carbohydrate metabolism suggesting resetting towards NADPH production (Godon *et al*., 1998). NADPH is known to be the co-factor for enzymes involved in antioxidant defence like glutathione reductase (EC 1.8.1.7) and thioredoxin reductase (EC 1.8.1.9) (Chae *et al*., 1994; Grant *et al*., 1996; Muller, 1996), hence, glycerol metabolism is suggested to be directly implicated in oxidative stress protection. The enzymes found to be with induced production are the isoenzymes encoded by *DAK1*, *GPD1*, *GPP2*/*HOR2*, and the Ybr149wp gene product putatively identified as the enzyme glycerol dehydrogenase by sequence homology to the correspondent orthologue from *A. niger* (Norbeck and Blomberg, 1997; Godon *et al*., 1998). The open reading frame *YBR149w* has been demonstrated to encode D-arabinose dehydrogenase (EC 1.1.1.117) with NADPH as co-factor (Kim *et al*., 1998), which is in agreement with the general resetting of carbohydrate metabolism reported by Godon and co-workers (1998). The possibility of Ybr149wp to act as glycerol dehydrogenase remains to be demonstrated. The overall effect on glycerol metabolism by oxidative stress is, consequently, the simultaneous anabolism and catabolism of glycerol creating a cycle with continuous consumption of NADH and generation of NADPH. Accordingly, analysis of mRNA levels revealed increased transcriptional activity of *GPP2/HOR2* in cells treated with the superoxide producing agent paraquat or with hydrogen peroxide and increased sensitivity of *gpp1*/*rhr2gpp2*/*hor2* double mutants to superoxide (Påhlman *et al*., 2001a). Another glycerol-defective mutant was demonstrated to be abnormally susceptible to hydroxyl radical and the over-expression in this mutant of the gene encoding mannitol 1-phosphate dehydrogenase (EC 1.1.1.17) from *Escherichia coli*, causing high production of the polyol mannitol, reverts this high susceptibility to oxidative stress (Chaturvedi *et al*., 1997). However, this reversion was explained to be through hydroxyl radical scavenging by mannitol, which is suggested to occur with glycerol as well.

It is known that oxidative stress response in yeast involves the induction of transcription of at least *TRX2*, encoding thioredoxin and *TRR1*, encoding thioredoxin reductase, by the transcription factors Yap1p and Skn7p (Schnell *et al*., 1992; Kuge and Jones, 1994; Krems *et al*., 1995; Krems *et al*., 1996; Morgan *et al*., 1997). Nevertheless, other genes are activated by these transcription factors in response to oxidative stress, although cooperation between these regulatory proteins has not been demonstrated for most of the cases. Another example is the gene *GSH1*, encoding glutamyl cysteine synthetase (EC 6.3.2.2) the first enzyme in glutathione biosynthesis pathway, which is regulated by Yap1p being, its expression induced in response to hydrogen peroxide (Stephen and Jamieson, 1997). In a global approach, by two-dimensional gel electrophoresis in mutant cells affected in *YAP1* or *SKN7* genes, only *DAK1*, among the genes of glycerol metabolism, was demonstrated to be dependent on Yap1p and Skn7p for expression (Lee *et al*., 1999a). Hence, expression of *GPD1* and *GPP2*/*HOR2* in oxidative stress response remains to be elucidated. Interestingly, from the H_2O_2 stimulon identified previously (Godon *et al*., 1998), Yap1p controls approximately half of the proteins and Yap1p and Skn7p simultaneously control approximately half of this group. So, two subsets of the Yap1p regulon are identified and constitute a remarkable distinction of genes encoding antioxidant-scavenging enzymes (Skn7p-dependent genes) from genes encoding enzymes of metabolic pathways for biosynthesis of the main reducing agents glutathione and NADPH (Skn7p-independent genes) (Lee *et al*., 1999b). Yet, several genes of unknown function are involved in oxidative stress response, suggesting new mechanisms of antioxidant cellular activity and new

mechanisms of transcriptional control of genes with unknown oxidative stress-driven regulation like *GPD1* and *GPP2*/*HOR2*.

The striking observation that five target genes of the repressor Sko1p, a mediator of the HOG (High Osmolarity Glycerol) signal transduction pathway (Brewster *et al*., 1993), are known or predicted to be involved in oxidative damage repair has caught the attention to the possibility of overlap or even interplay of osmotic and oxidative stress responses (Rep *et al*., 2001). These genes encode oxireductases and the correspondent disruption mutants of *AHP1*, *GLR1*, and *SFA1* were demonstrated to be hypersensitive to oxidative stress (Lee *et al*., 1999b; Muller, 1996; Wehner *et al*., 1993). Moreover, the induction of expression of these genes is mediated by Yap1p transcription factor (Rep *et al*., 2001). One of the possibilities to explain the osmotic stress-driven regulation of genes involved in oxidative damage repair implicates directly glycerol metabolism. Under osmotic stress, the high metabolic flux towards glycerol production (see Role in osmoregulation) could cause NAD^+ surplus produced in the reduction of dihydroxyacetone phosphate by NADH-dependent glycerol 3-phosphate dehydrogenase. The redox unbalance generated by depletion of reduction equivalents in the form of NADH may be perceived as oxidative stress by the cell (Rep *et al*., 2001). These unexpected features of interplay of stress responses suggest that adaptation to stressful conditions are much more complex than anticipated and the involvement of specific stress response mechanisms like glycerol accumulation in osmoadaptation might be common for many other stress conditions.

Role in heat-shock response

A protective role of glycerol against heat shock has been reported due to temperature sensitivity of mutant strains unable to carry out its synthesis like *gpd1gpd2* and *gpp1gpp2* (Siderius *et al*., 2000). Glycerol production in heat shock response was confirmed by determination of intracellular concentration, which was increased at 37˚C. Consistently, *hog1* mutant strains, affected in transcriptional activation of *GPD1* induced by osmotic stress, are able to accumulate glycerol as the wild type when grown at 37˚C. In addition, the characteristic osmosensitive phenotype of these mutants is suppressed by growth at 37˚C. The gene encoding the key enzyme in glycerol production for osmoregulation *GPD1* was found to be transcriptionally induced by heat shock at 42°C (Rep *et al*., 1999a), suggesting that this gene might be important as well for glycerol production in heat shock response.

External glycerol in media of cultures of *gpd1gpd2* and *gpp1gpp2* mutants causes suppression of the temperature sensitivity phenotype. In a similar experiment, addition of glycerol to the culture medium allowed recovery of the *gpd1gpd2* mutant strain but not of *gpp1gpp2*, which suggests an involvement of glycerol uptake, phosphorylation by a partially derepressed glycerol kinase and dephosphorylation by glycerol 3-phosphate phosphatase (Siderius *et al*., 2000). This way, intracellular glycerol accumulation is possible, allowing stabilisation of cellular structures. This is consistent with recent reports of reduced hypersensitivity to osmotic stress of the *ste11ssk2ssk22* mutant defective in the HOG pathway at 37°C by overexpression of *GPD1* and *FPS1* (Wojda *et al*., 2003). Interestingly, this effect has been attributed to glycerol through an indirect mechanism involving influence of signalling of the PKC pathway. Therefore,

response to heat shock and high osmolarity involves interplay of the HOG and PKC pathways, which regulate intracellular levels of glycerol (Wojda *et al*., 2003). Remarkably, a temperature sensitive phenotype has been reported in strains deleted in *GUP1* (Oelkers *et al*., 2000), which is involved in active glycerol uptake (Holst *et al*., 2000). A role of the putative glycerol active transporter Gup1p in heat shock response is therefore suggested by these observations.

The dual role of glycerol as compatible solute and as heat stress protective agent suggests interplay and cross-activation of stress responses. Both heat and salt shock can cause cross adaptation to heat, salt and freezing stress and when protein synthesis is blocked with cycloheximide, cross adaptation is still observed between heat and salt stresses although at lesser extent (Lewis *et al*., 1995). This is in accordance with a dual role for trehalose as well, acting in heat shock (De Virgilio *et al*., 1994; Hottiger *et al*., 1994) and in osmotic shock response (MacKenzie *et al*., 1988; Sharma, 1997; Hounsa *et al*., 1998). Nevertheless, the interplay between heat and osmotic responses might occur at a different level since the expression of the genes *HSP12* and *HSP26*, encoding heat shock proteins (Praekelt and Meacock, 1990; Petko and Lindquist, 1986; Susek and Lindquist, 1989), is induced with osmotic stress (Varela *et al*., 1992).

Involvement of glycerol in signal transduction pathways

The high osmolarity mitogen-activated protein kinase pathway

An osmotic challenge in yeast cells involves sensing and transduction of a signal to activate gene transcription, allowing to trigger physiological adaptation to the new environmental conditions. The major signal transduction pathway in osmotic stress response is the high osmolarity glycerol (HOG) mitogen-activated protein (MAP) kinase pathway (Brewster *et al*., 1993) and is similar to protein kinase cascades involved in cellular responses to different external stimuli like mating pheromones in yeast and growth factors in animals. Other signal transduction pathways have been found to regulate gene expression as well in osmotic stress response like the protein kinase A (PKA pathway) involved in responses to nutrient availability and several forms of stress (van der Plaat, 1974; Norbeck and Blomberg, 2000). Another signalling system was proposed involving phosphatidylinositol 3,5-bisphosphate as a second messenger (Dove *et al*., 1997) and another one with the participation of *SNF1* encoded protein kinase (Tsujimoto *et al*., 2000).

The HOG MAP kinase pathway has three sensor systems that trigger signal transduction through two input branches of this pathway (Fig. I.3). Stimulation has been achieved experimentally with several substances that do not cross the plasma membrane readily like glycerol, suggesting that the decreased turgor caused by the gradient across the plasma membrane is the signal perceived by the sensors (Tamás *et al*., 2000). The Sln1p sensor, similar to bacterial two-

component regulators (Ota and Varshavsky, 1993), acts co-ordinately with Ypd1p to inactivate Ssk1p under hypo-osmotic conditions (Maeda *et al*., 1994; Maeda *et al*., 1995; Posas *et al*., 1996). Hyper-osmolarity causes cessation of Ssk1p inactivation, which can, in turn, activate the MAPKKK elements of this cascade: Ssk2p and Ssk22p (Posas and Saito, 1998). These in turn activate the MAPKK Pbs2p that phosphorylates the MAPK Hog1p (Maeda et al., 1995; Posas and Saito, 1997). The phosphorylated form of Hog1p is mainly found in the nucleus where it can interact with transcription factors (Ferrigno *et al*., 1998; Reiser *et al*., 1999). The second branch of input signal includes a sensor system involving Sho1p and several other proteins forming a complex including upstream control elements like Cdc42p, Ste20p, and Ste50p and the MAPKKK Ste11p and MAPKK Pbs2p (Posas and Saito, 1997; O'Rourke and Herskowitz, 1998; Raitt *et al*., 2000; Reiser *et al*., 2000). Interestingly, the membrane-bound protein Sho1p is involved in HOG pathway activation by heat shock (Winkler *et al*., 2002), which constitutes a remarkable feature of the HOG signalling pathway suggesting that glycerol production is required for heat stress response. This is in agreement with the osmosensitive phenotype of *gpd1gpd2* mutants (Siderius *et al*., 2000) and the transcription induction of *GPD1* by heat shock (Rep *et al*., 1999b). A third osmosensor, encoded by *MSB2* was identified acting in parallel with the Sho1p sensor with which is partially redundant in activation of Ste11p (O'Rourke and Herskowitz, 2002). Another activation mechanism has been proposed for the HOG pathway that would phosphorylate Pbs2p under severe salt stress (Van Wuytswinkel *et al*., 2000).

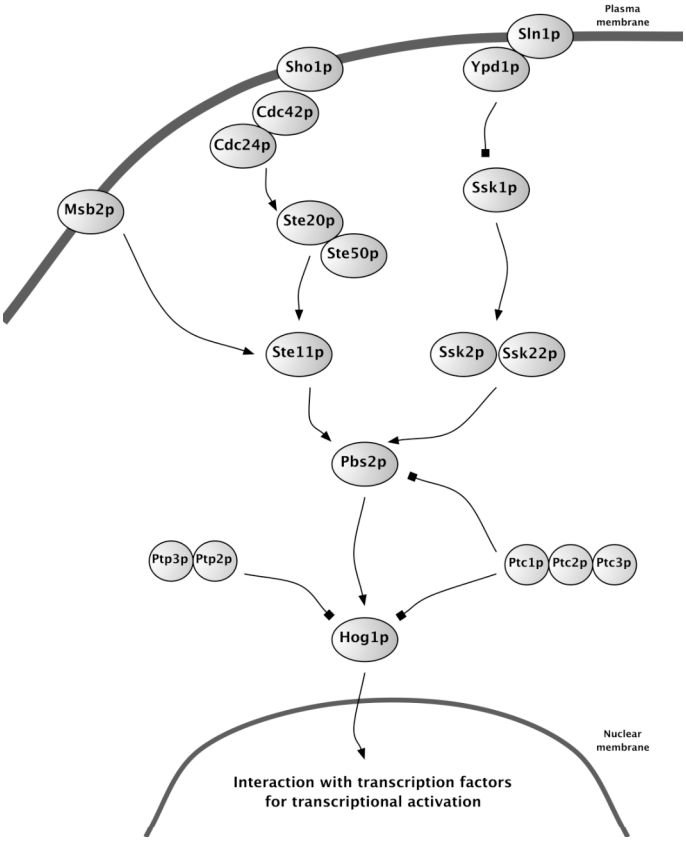

Figure I.3. The HOG signalling pathway of *Saccharomyces cerevisiae* (adapted from Hohmann, 2002).

Extragenic suppressors of *sln1* mutants with constitutive activation of the HOG pathway allowed the identification of negative regulators. The phosphotyrosine phosphatase Ptp2p was demonstrated to dephosphorylate the activated form of Hog1p (Ota and Varshavsky, 1992; Maeda *et al*., 1994) and the simultaneous disruption of *PTP2* and *PTP3* causes constitutive Hog1p phosphorylation even in the absence of osmotic stress (Wurgler-Murphy *et al*., 1997; Jacoby *et al*., 1997). In addition, Ptp2p and Ptp3p prevent hyperactivation of Hog1p in heat shock-activated HOG signalling pathway (Winkler *et al*., 2002). The action of these phosphatases has been suggested to be in connection with localisation of Hog1p since overexpression of *PTP2* results in hog1p nuclear retention while overexpression of *PTP3* results in Hog1p retention in the cytoplasm (Mattison and Ota, 2000). Type 2C serine/threonine phosphatases, Ptc1p, Ptc2p, and Ptc3p were found to act as negative regulators of the HOG pathway as well by dephosphorylation of Hog1p (Maeda *et al*., 1994; Warmka *et al*., 2001; Young *et al*., 2002a). The observation that *gpd1gpd2* and *gpp1gpp2* mutants, with impaired glycerol synthesis, have prolonged phosphorylation of Hog1p instead of the transient nature in wild type the strain, suggests an influence of the internal glycerol in the HOG pathway activation (Siderius *et al*., 2000). This is consistent with the Sln1p activation by autophosphorylation in an *fps1* mutant strain (Tao *et al*., 1999). The proposed mechanism for this effect involves the elevated content of glycerol in *fps1* mutants that would mimic hypo-osmotic conditions and hence stimulation of the Sln1p sensor. The notion that sensors might be activated by a stimulus different than interaction with an external osmolyte is further supported by the observation that the HOG pathway is stimulated by changes in membrane tension (Tamás *et al*., 2000).

Transcriptional regulators interacting with promoters of target genes mediate the HOG pathway signalling and include proteins of distinct families that may activate or inactivate transcription (recently reviewed by Hohmann, 2002). The transcription factor Sko1p/Acr1p is a repressor of *ENA1*, encoding a P-type ATPase involved in sodium and lithium extrusion (Haro *et al*., 1991) and activation of a set of genes encoding oxireductases (Rep *et al*., 2001). The action of this factor involves binding at the promoter to the cAMP response element (CRE), an upstream repressing sequence similar to mammalian cyclic AMP response elements (Proft and Serrano, 1999). Another transcription factor is Gcn4p which binds a CRE element for activation of transcription of *HAL1*, a gene implicated in ion homeostasis (Gaxiola *et al*., 1992). Interestingly, this gene is under the coordinated regulation of Gcn4p and the repressor Sko1p/Acr1p via CRE elements in its promoter (Pascual-Ahuir *et al*., 2001). Moreover, Gcn4p is involved as well in transcriptional regulation of genes encoding enzymes of biosynthesis of amino acids and is involved in the response to amino acid starvation (reviewed by Hinnebuch, 1986). The activators Msn2p and Msn4p bind stress responsive elements (STRE) in order to induce transcription of genes required for general stress response in yeast (Schüller *et al*., 1994; Martinez-Pastor *et al*., 1996; Rep *et al*., 2000). The transcriptional activator Skn7p is the mediator of a Mcm1p signalling pathway which shares Sln1p-dependent regulation with the HOG signalling pathway (Brown *et al*., 1994; Yu *et al*., 1995; Fassler *et al*., 1997; Ketela *et al*., 1998). When HOG signalling is inactivated, the Mcm1p pathway is activated (Fassler *et al*., 1997), suggesting coordinated functioning of both pathways. Due to the demonstrated involvement of Skn7p in oxidative stress response (Li *et al*., 1998a; Lee *et al*., 1999a), this coordinated action could involve this one and osmotic stress response. The Msn1p transcriptional activator involved in iron-limited growth, pseudohyphal growth,

and in invertase regulation (Lambrechts *et al*., 1996) is another mediator of osmotic stress-induced gene expression (Rep *et al*., 1999b). The transcriptional regulator Sgd1p, to which converge, in osmotic stress response, the HOG pathway and a pathway that includes phospholipase C encoded by *PLC1* (Lin *et al*., 2002), was identified previously as a multicopy partial suppressor of *pbs2* and *hog1* mutants (Akhtar *et al*., 2000). The activator Hot1p has been demonstrated to regulate transcription of target genes of the HOG pathway as well (Rep *et al*., 1999b; Rep *et al*., 2000). Recently, a new transcription factor Smp1p was identified as being phosphorylated by Hog1p and acting downstream this MAPK (de Nadal *et al*., 2003). In addition, evidence was obtained pointing to a role in stationary phase for HOG pathway mediated by Smp1p.

HOG pathway-dependent induction of transcription affects genes involved in glycerol production, in accordance to the physiological effects of osmotic stress, and genes of many other functional categories pointing to an involvement in other cellular processes (Rep *et al*., 2000). One of the most important targets is *GPD1* encoding the key enzyme in glycerol biosynthesis glycerol 3-phosphate dehydrogenase (Albertyn *et al*., 1994b; Akhtar *et al*., 1997). The transcriptional regulators known to bind promoter regions of *GPD1* and *GPP2*/*HOR2* for HOG-dependent transcriptional activation are Hot1p, Msn1p, Msn2p, and Msn4p (Rep *et al*., 1999b). The activator Sgd1p has been demonstrated to regulate only *GPD1* (Akhtar *et al*., 2000) and, for *DAK1*, evidence of HOG-dependent induction mediated by Msn2p and Msn4p has been reported since mRNA levels are decreased under salt stress in *hog1* and in the *msn2msn4* double mutant (Rep *et al*., 2000). This is consistent with the glycerol futile cycle under osmotic stress, allowing to consume the ATP surplus and to regenerate inorganic phosphate (Blomberg, 2000). Interestingly, the levels of *GPP1* mRNA were observed to be under control by the HOG-pathway (Rep *et al*., 2000), although in less extent than *GPP2*, which is in agreement with the lack of osmosensitive phenotype observed in *gpp2* mutants (Påhlman *et al*., 2001a). Other transcription factors are likely to bind promoters of genes of glycerol metabolism. The observation of the influence of *TPS1,* encoding a subunit of the trehalose synthase complex, on *GPD1* transcription and glycerol production has been explained as the activity of Tps1p as a transcription factor or by the interaction with known or unknown transcription factors of the HOG pathway (Hazell *et al*., 1997).

Only a minor proportion of genes upregulated in osmotic stress depends exclusively on the HOG pathway (Rep *et al*., 2000). This suggests the convergence of multiple signalling pathways for the expression of almost all genes required for osmotic stress response, in which are included *GPD1* and *GPP2* (Rep *et al*., 1999b; Rep *et al*., 2000). In this respect it is interesting to note that the possibility of multiple signalling pathways targeting *GPD1* and *GPP2* is in agreement with biochemical evidence of intervention of glycerol in several physiological roles and stress responses.

The importance of glycerol in osmotic stress response is corroborated in genome-wide transcription analysis by microarrays performed in the *gpd1gpd2* mutant strain (Yale and Bohnert, 2001). In the absence of glycerol production in the case of the *gpd1gpd2* mutant, the transcript profile under osmotic stress is rather different than the one of the wild type. A major difference is the activation of transcription of genes of the pheromone response pathway, suggesting a connection with the HOG pathway. Cross talk between these signalling pathways should be possible since the elements Ste20p, Ste50p and Ste11p are common.

However, single mutants affected in *PBS2* and *HOG1* allowed activation of the pheromone response pathway, suggesting tight regulation of cross talk (O'Rourke and Herskowitz, 1998). On the other hand, when target genes are inoperative like *GPD1* and *GPD2* a signal overflow might be channelled to pathways sharing protein kinases (Hohmann, 2002), which can be exacerbated by deficient feedback regulation due to absence of glycerol to recover turgor.

Convergence of distinct signalling might occur at the promoter of a given gene, suggesting potentiation of transcription activation. An example of such convergence is the *GPD1* transcriptional regulation by Rap1p (repressor activator protein 1) implicated in many cellular processes like silencing of mating-type genes, telomere function and structure, and stimulating meiotic recombination (reviewed by Shore, 1994). This transcriptional factor was demonstrated to be an important activator responsible for both basal and salt-induced transcriptional activities of *GPD1* promoter via a proposed interaction with Hot1p and/or Msn1p (Eriksson *et al*., 2000). Further evidence of convergence of different signalling pathways for regulation of *GPD1* expression has been obtained by analysis of *hog1* mutants, which display delayed induction of *GPD1* transcription to levels of mRNA moderately lower than the wild type (Rep *et al*., 1999a). Identification of signalling pathways, other than HOG MAPK, influencing *GPD1* expression and hence synthesis of glycerol has not been reached. However, recently, a signal transduction pathway that includes phospholipase C (EC 3.1.4.11) encoded by *PLC1* was proposed to function in parallel with the HOG pathway and that would converge on the transcription factor Sgd1p to activate *GPD1* expression (Lin *et al*., 2002). The gene *PLC1* encodes a phospholipase that catalyses hydrolysis of phosphatidylinositol 4,5-bisphosphate, producing the second messengers inositol 1,4,5-trisphosphate and 1,2-diacylglycerol involved in nutritional and stress responses (Flick and Thorner, 1993). So, considering that glycerol is a precursor of lipid biosynthesis, the possibility of interaction between the HOG pathway and a signalling pathway implicated in lipid metabolism should not be disregarded. Evidence for another signalling pathway come from the observation that the Ssn6p/Cyc8p-Tup1p repressor complex of the glucose repression pathway influences *GPD1* transcription among other HOG-dependent and HOG-independent genes involved in osmotic stress response (Márquez *et al*., 1998).

Interestingly, the existence of isoenzymes for each step of glycerol anabolism via glycerol 3-phosphate, encoded by homologous genes with different regulation denotes tight metabolic control and a broad intervention in cellular metabolism. Another level of complexity is conferred by some of the transcription factors that mediate HOG pathway signalling which are implicated in other cellular processes of environmental stress conditions or starvation. The example of the transcription factors Msn2p and Msn4p (Martinez-Pastor *et al*., 1996) is consistent with this possibility given the wide range of genes implicated with distinct metabolic pathways with which they interact. As has been pointed for direct involvement of enzymes of glycerol metabolism in several stress conditions, the overlap of different signalling pathways indicate the cross adaptation for different stresses. Another possibility is the appearance of a secondary stress caused by metabolic disturbance of an environmental stress.

Interplay between signalling pathways contributes to further levels of regulation of signalling and transcription. A well-documented example is the RAS-cAMP-mediated repression of Msn2p and Msn4p (Boy-Marcotte *et al*.,

1998; Görner *et al*., 1998). This repression has been reported to occur in a variety of cellular processes like diauxic transition (Boy-Marcotte *et al*., 1998; Garreau *et al*., 2000), general stress response (Görner *et al*., 1998), growth and glycogen accumulation (Smith *et al*., 1998), heat shock (Garreau *et al*., 2000) and osmotic stress (Norbeck and Blomberg, 2000). Interestingly, protein kinase A, a target of cAMP the central messenger of the RAS-cAMP pathway (reviewed by Thevelein, 1994 and Thevelein and de Winde, 1999), strongly represses genes influenced by salt stress, excluding those involved in glycerol metabolism: *GPD1*, *GPP2* and *DAK1* (Norbeck and Blomberg, 2000). The reason for this striking distinction for regulation remains unclear however, it suggests that glycerol metabolism is regulated in a coordinated fashion, despite the exception of *GCY1*, encoding glycerol dehydrogenase, that was found to be dependent on protein kinase A activity.

A second signal transduction pathway, the PKC pathway, that senses osmotic changes has been identified and is distinct from the HOG pathway by the fact that it is activated through hypo-osmotic shock (Davenport *et al*., 1995). The PKC1 pathway is a MAP kinase pathway that is implicated in cell wall construction and therefore in cell wall integrity and morphogenesis and mutants affected in genes encoding elements of this pathway display the characteristic phenotype of cell lysis (Levin and Bartlett-Heubusch, 1992). Consistently with an involvement in cell integrity is the phenotype exacerbation at high temperatures and reversion with osmotic stabilisers (Levin and Bartlett-Heubusch, 1992; Paravicini *et al*., 1992). Remarkably, overexpression of *LRE1*, *HLR1* and *WSC3*, known to affect cell wall composition (Lai *et al*., 1997; Verna *et al*., 1997; Alonso-Monge *et al*., 2001), suppress osmotic sensitivity induced by high temperatures of mutant strains affected in the HOG pathway and the temperature-induced cell lysis of PKC pathway mutants (Alonso-Monge *et al*., 2001). These observations strongly suggest close interplay between these signal transduction pathways through the existence of common targets. In agreement with this is the hypersensitivity to cell wall-degrading enzymes (Alonso-Monge *et al*., 2001) and the resistance to the chitin-interacting drug calcofluor (García-Rodriguez *et al*., 2000) of HOG pathway mutants. These authors further observed a striking increase in salt tolerance and glycerol accumulation by treatment with calcofluor without activation of the HOG pathway and independently of functional *GPD* genes. Nevertheless, tight regulation of signalling in order to allow specificity of activation is suggested by the fact that these effects are only observed in mutant strains and by the possibility of a function of cross talk-prevention by the protein phosphatases Ptp2p and Ptp3p. In strains deleted in *PTP2* and *PTP3*, *HOG1*-dependent lethality occurs when a hyperactive *MKK1* allele is present instead of the wild type MAPKK of the PKC pathway (Winkler *et al*., 2002).

The possibility of interplay between the HOG pathway and the pseudohyphal development pathway (also known as filamentation/invasion pathway) has been reported by the observation that the pseudohyphal development pathway MAPK, Kss1p, is phosphorylated in mutants affected in *PBS2* (Davenport *et al*., 1999). Another evidence is the phenotype of cellular projections formation induced by osmotic shock in these mutants, which is absent in the pseudohyphal development pathway-deficient mutant strains *kss1* and *ste7* (Davenport *et al*., 1999). Consistently, in *hog1* and *pbs2* mutants the recovery from osmotic stress is made with the production of a single new bud despite the existence of other buds prior to osmotic shock (Brewster and Gustin, 1994). This phenotype was explained with the requirement of the HOG pathway for repositioning of the actin

cytoskeleton in order to continue development of existing buds. Interestingly, a mutation in *KSS1* was selected as suppressor of the osmosensitive phenotype of the *hog1* deletion mutant with restoring of *GPD1* induction (Lee *et al*., 2002). Surprisingly, the presumable higher content of glycerol under these conditions is not consistent with the observed phenotype of temperature sensitivity in the presence of osmotic stress and glucose.

Chapter II

Genomic library screening for cloning genes involved in glycerol active uptake

Introduction

Osmotic stress induced by salt causes *Saccharomyces cerevisiae* cells to accumulate glycerol, acting as compatible solute, in order to balance water potentials inside the cells in relation to the surrounding medium (Brown, 1990). In *S. cerevisiae*, the key enzyme for glycerol synthesis is glycerol 3-phosphate dehydrogenase (EC 1.1.1.8) encoded by the gene *GPD1* which was cloned independently by Larsson and co-workers (1993) and Albertyn and co-workers (1994b). The expression of this gene was found to be under induction of osmotic stress (Albertyn *et al.*, 1994b; Ansell *et al.*, 1997) and the respective mutants showed an osmotic stress sensitivity phenotype with very low amounts of intracellular glycerol (Albertyn *et al.*, 1994b).

Among the enzymes of glycerol metabolism in *S. cerevisiae*, Gpd1p, Gpp2p, Dak1p, and the putative glycerol dehydrogenase Gcy1p (aldo-keto reductase; EC 1.1.1.156) were found to have increased rate of synthesis by two-dimensional polyacrylamide gel electrophoresis analysis of adapted cells to salt stress (Norbeck and Blomberg, 1997; Norbeck and Blomberg, 2000). These authors have complemented the protein expression study with Northern analysis and found a correlation between the mRNA levels with the protein synthesis rate for the correspondent enzymes. In addition, analysis of global transcriptional response to salt stress by microarrays (Rep *et al.,* 2000; Posas *et al.,* 2000; Yale and Bohnert, 2001) confirmed *GPD1* and *DAK1* to be up-regulated at transcriptional level as well. Although, the results of these three studies do not coincide in salt stress induced transcription of *GPP1*, *GPP2*, and *GPD2*, which are considered up-regulated or unresponsive, the data available allows to draw a basic picture of increased synthesis of glycerol upon salt stress.

The hypothesis that intracellular accumulation of glycerol in *S. cerevisiae* is done at the expense of increased synthesis, under salt stress, is consistent with the activity of the glycerol channel Fps1p. This protein was reported to be responsible for glycerol facilitated diffusion (Sutherland *et al.*, 1997) and, hence, being unable to promote intracellular accumulation. The involvement in lipid biosynthesis was reported by the same authors so that plasma membrane permeability may be, which could contribute to glycerol retention. On the other hand, Luyten and co-workers (1995) suggested an involvement of *FPS1* in glycerol synthesis by a mechanism that is still not understood. More recently, Tamás and co-workers (1999) have reported that Fps1p is involved in glycerol efflux rather than uptake and that its expression is mainly by turgor changes (Tamás *et al.*, 2000). These observations suggest adaptation to changes of osmolarity through opening/closure of the channel in order to regulate intracellular levels of glycerol. This mode of action involves the Fps1p N-terminal domain close to the first transmembrane domain, since mutants truncated in this region present constitutive glycerol efflux and increased osmotic sensitivity (Tamás *et al.*, 1999).

Evidence for an active transport system for glycerol has been presented by Lages and Lucas (1997), who suggested a physiological role connected to respiratory/gluconeogenic conditions for this transport system. As an active transport system, this glycerol transporter can accumulate the substrate against

gradient however, since no active uptake and intracellular accumulation with the radiolabelled substrate was ever detected in glucose-grown cells with NaCl 1 M, its activity could not be attributed to osmotic stress response. Earlier, Albertyn and co-workers (1994b) have suggested the existence of an active transport system for glycerol based on the decrease of external glycerol in adapting *gpd1hog1* double mutant to salt stress which produces small amounts of glycerol. Consistently, Luyten and co-workers (1995) have observed that *fps1* mutants present more glycerol uptake than the wild type parental strain under salt stress, which can be explained by the assumption of an additional transport system.

The striking difference between the observations of Lages and Lucas (1997), that the active transport system for glycerol is not connected to osmotic stress, with those of Albertyn and co-workers (1994b) and of Luyten and co-workers (1995) that it actually should be involved in this stress, suggests a complex regulation of glycerol transport across the plasma membrane. It should be noted, however, that indications of involvement of a glycerol active transport system in osmotic stress have been found in mutant strains with impaired synthesis of glycerol and not in wild type strains where synthesis can contribute in very high extent to intracellular accumulation (Luyten *et al.*, 1995). Taken together, experimental data support the idea that glycerol is mainly accumulated through high rates of synthesis. Plasma membrane-associated transport systems would contribute to glycerol accumulation by decreasing plasma membrane permeability and, presumably, by a mechanism of fine-tunning regulation of intracellular glycerol content.

The intracellular accumulation of glycerol in osmoregulation is a well-known mechanism in *S. cerevisiae* cells. However, there is experimental evidence suggesting that this phenomenon is much more complex and intertwined with the adaptation to other types of stress. In fact, global approaches like two-dimensional polyacrylamide gel electrophoresis have detected several proteins with increased and decreased rate of synthesis by osmotic shock and by adaptation to osmotic stress (Varela *et al.,* 1992; Blomberg, 1995; Norbeck and Blomberg, 1996; Norbeck and Blomberg, 1997). The existence of induced and repressed protein expression is rather interesting as it suggests the mobilization of some physiological mechanisms while others are shut down. So, these global approaches, not only, uncover the involvement of many uncharacterised genes, they also show the complexity of osmoregulation. On the other hand, interplay between different types of stress was found in these studies by detection of increased synthesis rates of heat shock proteins Hsp12p, Hsp26p (Varela *et al.*, 1992), and Hsp104p (Blomberg, 1995) and the cross adaptation to heat, freezing and salt stresses by heat-shocked and salt-shocked cells (Lewis *et al*., 1995).

Besides intracellular glycerol accumulation, other physiological processes contribute to surviving under conditions of osmotic stress caused by sodium chloride. To prevent the toxicity of Na^+ ions, mechanisms controlling intracellular homeostasis must act mainly by extrusion of this ion and accumulation of K^+ ions resulting in high levels of K^+/Na^+ ratio. This homeostasis has been demonstrated to be dependent on calcineurin, which acts as regulator of expression of ion transporters and on their activity (Nakamura *et al*., 1993; Mendoza *et al*., 1994; Hirata *et al*., 1995; Danielsson *et al*., 1996). Other genes were implicated in ion homeostasis by regulating gene expression of cation transporters: *HAL1* (Gaxiola *et al*., 1992; Rios *et al*., 1997), *HAL3* (de Nadal *et al*., 1998), and *HAL4*

and *HAL5* (Mulet *et al*., 1999); or even by a novel function with the involvement of *SOP1* and *SOP2* genes (Larsson *et al*., 1998b). The actual cation transporters are the Na^+ extrusion systems *ENA1*/*PMR1* (Haro *et al*., 1991; Wieland *et al*., 1995) and *NHA1* (Banuelos *et al*., 1998) and the potassium transporters *TRK1* and *TRK2* (Ko and Gaber, 1991; Ramos *et al*., 1994). Mechanisms involved in cell proliferation were demonstrated to have a role in salt stress tolerance, such as actin synthesis and actin filaments organization for budding (Novick and Botstein, 1985; Chowdhury *et al*., 1992; Brewster and Gustin, 1994; Srinivasan *et al*., 1998), and karyogamy and cell fusion (Schoch *et al*., 1997; Philips and Herskowitz, 1997). Mutant strains were reported to be associated to impairment in salt stress tolerance: plasma membrane ATPase (McCusker *et al*., 1987), vacuolar biogenesis (Banta *et al*., 1988), and the protein kinase C encoding gene *PKC1* (Shimizu *et al*., 1994). On the other hand, the nucleotidase gene *HAL2*, that increases salt tolerance in high dosage, was found to be allelic to *MET22,* a gene encoding a methionine biosynthesis enzyme (Gläser *et al*., 1993; Murguía *et al*., 1996).

The evidence of novel genes involved in osmotic stress response comes, as well, from the obtainment of mutants in uncharacterised genes with altered phenotypes regarding osmotic stress. Gaxiola and co-workers (1996) isolated a spontaneous mutant with a phenotype of increased halotolerance together with low ratio of Na^+ and K^+ intracellular concentrations, high polysomal stability, and salt stress-dependent altered protein expression. These pleiotropic effects caused by only one nuclear dominant mutation are consistent, as mentioned above, with the complexity of the osmotic stress response. In a different work for isolation of osmosensitive mutants created by random mutagenesis, Brüning and co-workers (1998) obtained several mutants with little or no increase in intracellular glycerol when exposed to osmotic stress. These findings lead the authors to conclude that a wide spectrum of genes is actually involved in osmotic stress response. The fact that mutated genes can cause osmosensitive or osmotolerant phenotypes can be explained by a possible role of these genes as regulators of expression of other genes or as modulators of enzymatic activity in physiological mechanisms necessary for adaptation to osmotic stress.

The existence of an active transport system for glycerol in *S. cerevisiae* without being involved in osmoregulation is surprising if we take in consideration the examples of *Zygosaccharomyces rouxii* (van Zyl *et al*., 1990), *Pichia sorbitophila* (Lages and Lucas, 1994; Oliveira and Lucas, 1996) and the demonstrated connection between halotolerance and glycerol active uptake in many yeast species (Lages *et al*., 1999). It is well known that fermentative metabolism of *S. cerevisiae* causes redox imbalance with depletion of NAD^+, which is produced by synthesis of glycerol in the reaction of reduction of glyceraldehyde 3-phosphate to glycerol 3-phosphate (van Dijken and Scheffers, 1986). During glucose fermentation, glycerol production becomes very high in exponentially growing yeast cells, making of glycerol one of the most abundant fermentation products besides ethanol. Therefore, the activity of an active transport system for glycerol could not be as essential in osmoregulation as for the uptake of glycerol for respiration when external glucose is depleted, which is consistent with gene expression data mentioned above. Accordingly, a *gpd1gpd2* double mutant, that cannot synthesise glycerol, presents an osmosensitive phenotype (Ansell *et al*., 1997). However, it is reasonable to assume that in this mutant strain, an active transport system for glycerol could contribute to osmotolerance provided the presence of glycerol in the medium.

The activity of this transporter would maintain a concentration gradient across the plasma membrane (Lages and Lucas, 1997) and, thus, this could be a good system for searching the gene encoding the glycerol active transporter of *S. cerevisiae*. The aim of the present work is to clone the gene encoding the glycerol active transporter by suppression of the low salt stress resistance phenotype conferred by *gpd1gpd2* mutations with a *S. cerevisiae* genomic library.

Materials and methods

Yeast and bacterial strains, media and growth conditions

Saccharomyces cerevisiae strain YSH642 (*MAT***a** *leu2-3, 112 ura3-1 trp1-1 his3-11, 15 ade2-1 can1-100 gpd1*::*TRP1 gpd2*::*URA3*) (kindly provided by Stefan Hohmann) derived from strain W303-1A (Thomas and Rothstein, 1989), was used as recipient for the genomic library. As control strains, besides W303-1A, YSH294 (isogenic to W303-1A but *fps1::LEU2*) and YSH7.88.-3D (isogenic to W303-1A but *fps1::LEU2 gpd1*::*TRP1 gpd2*::*URA3*) were used as well. Solid rich medium for maintenance of strains was YPDA [1% (w/v) yeast extract; 2% (w/v) peptone; 2% (w/v) glucose; 2% (w/v) agar]. Liquid rich medium for preparation of cells for transformation was YPD. Growth was performed at 30ºC, 160 rpm, and monitored by spectrophotometry at 600 nm. Selection of transformants was performed in synthetic medium agar plates (YNBDATrp$^-$Ura$^-$ Leu$^-$) prepared with 0.67% (w/v) yeast nitrogen base (YNB) without amino acids (DIFCO), 2% (w/v) glucose, 40 mg/l adenine, 40 mg/l L-histidine, and 2% (w/v) agar. Phenotype selection medium agar plates were prepared with synthetic medium (YNBDATrp$^-$Ura$^-$Leu$^-$) supplemented with 1.4 M NaCl and 50 mM glycerol (YNBDATrp$^-$Ura$^-$Leu$^-$ + 1.4 M NaCl + 50 mM glycerol).

To determine the osmotic tolerance of the recipient strain compared to the parental strain, osmotic sensitivity tests were done using NaCl (0.7 M, 1 M, 1.2 M; 1.4 M; 2 M; 2.2 M) and sorbitol (1.5 M; 1.7 M; 2 M; 2.5 M; 3 M; 3.5 M; 4 M) as stress agents in rich medium (YPDA) and synthetic medium (YNBDA), with or without 50 mM glycerol. Inocula were prepared from, either, 2 days-grown single isolated colonies in YPDA plates and suspension in 100 µl sterile deionised water or from 10^{-1} to 10^{-5} dilutions of overnight-grown cultures on YPD. To inoculate plates, 10 µl drops of each suspension were used and incubation was at 30ºC in sealed plastic bags for 7 days.

Escherichia coli strain DH5α [F- ϕ80d*lac*ZΔM15 Δ(*lac*ZYA-*arg*F)U169 *end*A1 *rec*A1 *hsd*R17(r_k^- m_k^+) *deo*R *thi*-1 *pho*A *sup*E44 λ-*gyr*A96 *rel*A1] was used for maintenance of genomic library and amplification of isolated clones. Bacterial cells were grown in LB liquid medium (10 g/l tryptone, 5 g/l yeast extract, 5 g/l NaCl, 1 N NaOH to adjust to pH 7.5) or LB agar plates with additional 2% (w/v) agar. Cultivations were done at 37ºC with agitation at 200 r.p.m. For plasmid maintenance and amplification, growth was performed in LB supplemented with

100 µg/ml ampicillin (LBamp), for solid agar plates, and 50 µg/ml ampicillin, for liquid medium.

All biological materials were kept at -80ºC for long-term storage. Yeast strains and yeast transformants were stored in 50% (v/v) glycerol, directly from saturated liquid culture. Bacterial strain and transformants were prepared for storage diluting 1 ml of saturated liquid cultures in 1 ml of glycerol solution [65% (v/v) glycerol, 0.1 M $MgSO_4$, 0.025 M tris·HCl, pH 8.0].

Genomic library and bacterial transformation

The genomic library (kindly provided by Margarida Casal and Carlos Gancedo) was originally prepared with partially *Sau*3AI digested genomic DNA with average size of 5 kbp from *S. cerevisiae*, inserted at the *Bam*HI restriction site of YEp13 shuttle vector (Table II.I).

Table II.I. Main features of YEp13 shuttle vector (adapted from Ausubel *et al*., 1996).

Size	*E. coli* replicon	Yeast replicon	Phenotypes selectable in *E. coli*	Phenotypes selectable in yeast	Reference
10.7 kb	pMB1	2µm	Ap^r, Tet^r, $LeuB^+$	Leu^+	Broach *et al*., 1979

For genomic library amplification and molecular manipulation of clones, *E. coli* cells were transformed using a calcium chloride-based protocol (Sambrook *et al*. 1989). Competent cells were prepared by growing in LB liquid medium and harvesting at mid-exponential phase by centrifugation 5 min, 1600x*g*, 4ºC. The cell pellet was resuspended in half volume of ice-cold 50 mM calcium chloride and placed on ice for 20 min. After new centrifugation under the same conditions, the pellet was resuspended in one tenth of initial culture volume of ice-cold 100 mM calcium chloride and kept on ice until use or frozen in liquid nitrogen and stored at -80ºC for long-term preservation. Each transformation was done with 100 ng of plasmid DNA and 100 µl of competent cells, except for the negative control in which the DNA was omitted. The mixture was incubated on ice for 60 min and cells were then heat-shocked at 42ºC for 2 min. Afterwards, 1 ml LB medium was added followed by 60 min incubation at 37ºC, 100 r.p.m. Selection of transformants was done with agar plates containing LB medium supplemented with ampicillin.

Analysis of DNA

Concentration and detection of protein and/or phenol contamination was done by spectrophotometry at 260 nm and 280 nm. A_{260}/A_{280} ratios were calculated and compared to reference values (Ausubel *et al.*, 1996), and when samples had ratios between 1.8 and 2.0, were considered free of protein and phenol. Integrity and RNA contamination was determined by agarose gel electrophoresis according to Ausubel *et al.* (1996) and Sambrook *et al.* (1989). Samples were prepared with 1-5 µg DNA and loading buffer [20% (w/v) ficoll 400; 0.1 M Na_2EDTA, pH 8.0; 1.0% (w/v) SDS; 0.25% (w/v) bromophenol blue] to a final

volume of 20 µl. Gels were prepared with 0.8% (w/v) agarose (Agarose MP, Roche), TAE buffer (40 mM tris·acetate, pH 8.5; 2 mM EDTA), and 0.5 µg/ml ethidium bromide. One lane of the gel was loaded with DNA molecular weight marker (Molecular Weight Markers IV, X, or XIV; Roche) in order to run simultaneously with samples and the electric field applied was of 1-10 V/cm. Gels were visualised under UV light and photographed. For samples free of contaminants such as proteins, phenol, and RNA, concentration was calculated by the formula [DNA]µg/µl = A_{260} x dilution x 50.0 (Ausubel *et al.*, 1996)].

Transformation of Saccharomyces cerevisiae gpd1gpd*2 mutant strain*

Yeast transformation was done by electroporation according to Ausubel *et al.* (1996) and Becker and Guarente (1991). Cells were grown in YPD and harvested at OD_{600}=1.3 to 1.5, by centrifugation at 4000x*g*, 4ºC, and resuspended in 250 ml ice-cold sterile ultra-pure water. Three more washes were done by centrifugation under the same conditions and resuspension in, sequentially, 125 ml ice-cold ultra-pure sterile water, 10 ml ice-cold sterile 1 M sorbitol, and 250 µl ice-cold sterile 1 M sorbitol. In a pre-refrigerated microcentrifuge tube, 40 µl of cell suspension and 100 ng DNA (in a volume minor than 5 µl) were mixed and kept on ice for 15 min. A negative control was included by omission of the DNA. The cell suspension was then transferred to an ice-cold 0.2-cm-gap electroporation cuvette (Gene Pulser® Cuvette, Bio-Rad) and placed in the electroporator (Gene Pulser® II with Pulse Controller II and Capacitance Extender II). Electroporation was made by a pulse of 1.5 kV, 25 µF, 200 Ω to cause a field strength of 1.5 kV for 5 msec. Immediately after electroporation, 960 µl ice-cold YNBD medium [0.67% (w/v) yeast nitrogen base (YNB) (DIFCO), 2% (w/v) glucose, 40 mg/l adenine, 40 mg/l L-leucine, 40 mg/l uracil] was added to the electroporation cuvette to gently resuspend the cells. Recovery of cells was done by incubation at 30ºC with gentle agitation (100 r.p.m.) for 60-80 min. Aliquots of 100 µl of this suspension were spread on selective agar plates (YNBDATrp$^-$Ura$^-$Leu$^-$) for genomic library screening and on rich non-selective agar plates in order to test viability (by comparison with plates inoculated with non-electroporated cells). Plates were incubated at 30ºC for 3 to 6 days.

In tests to confirm coacquisition of plasmid and phenotype, yeast cells were transformed with the lithium acetate method as well (Ausubel *et al.*, 1996). Cells from a 300 ml culture grown up to OD_{600}=0.4 were harvested by centrifugation 5 min, 4000x*g* at room temperature and resuspended in 10 ml sterile ultra-pure water. The suspension was cetrifuged 5 min, 6000x*g* at room temperature and the pellet was resuspended in 1.5 ml buffered lithium solution [1 vol. 10x TE buffer, pH 7.5 (10 mM tris·HCl, pH 7.5; 1 mM EDTA pH 8.0) + 1 vol. 10x lithium acetate stock solution (1 M lithium acetate, pH 7.5 adjusted with acetic acid) + 8 vol. sterile ultra-pure water]. For each transformation, plasmid DNA (5 µg) was mixed with 200 µg salmon sperm DNA in a volume of 20 µl. Transformation was made with 200 µl yeast suspension, the mixture of DNAs, and 1.2 ml PEG solution [8 vol. 50% (w/v) PEG 4000 + 1 vol. 10x TE buffer, pH 7.5 + 1 vol. 10x lithium acetate stock solution] with incubation 30 min, 30ºC with agitation. Cells were heat shocked at 42ºC for 15 min, microcentrifuged at top speed, and resuspended in 200 µl 1x TE buffer. After complete resuspension, cells were

plated on plasmid-selective minimal medium and incubated at 30ºC for 2 to 5 days.

Genomic library screening

All transformants obtained were replica-plated from transformant selective agar plates (YNBDATrp$^-$Ura$^-$Leu$^-$) to phenotype selective agar plates (YNBDATrp$^-$Ura$^-$Leu$^-$ + 1.4 M NaCl + 50 mM glycerol) with incubation at 30ºC for 7 days in sealed plastic bags in order to prevent drying of media and over-concentration of salt. Positive clones were tested, again, for plasmid and phenotype (with and without 50 mM glycerol) in the same media used before for the screening, by making a suspension in water from isolated colonies and inoculating with 5 µl. Subsequently, the confirmed positive clones were tested, with inocula prepared as stated previously for osmotic sensitivity tests, for increased sodium chloride concentrations (2 M, 2.2 M, 2.5 M, and 3 M; with and without 50 mM glycerol) and to other osmostress agents: sorbitol (1.5 M, 1.7 M, 2 M, 2.2 M, and 2.5 M; with and without 50 mM glycerol) and sucrose (1 M, 1.5 M, and 2.1 M; with and without 50 mM glycerol).

The confirmation of the linkage between the plasmid and the stress phenotype, for each positive clone, was done with cosegregation tests, according to Rose and Broach (1991). Rich, non-selective medium (YPD) was inoculated with an isolated colony from each transformant, and was incubated at 30ºC, 160 r.p.m. for 18-24 h. From serial dilutions of the culture, an aliquot of a 10^{-5} dilution was spread on YPDA plate and incubated at 30ºC for 2 days. Colonies were replica plated to selective media to select for plasmid-bearing cells (YNBDATrp$^-$Ura$^-$Leu$^-$) and to select complemented transformants (YNBDATrp$^-$Ura$^-$Leu$^-$ + 1.4 M NaCl + 50 mM glycerol) and were incubated under usual conditions.

Simultaneously, clones were tested for coacquisition of plasmid and phenotype. Plasmids from positive clones were isolated according to Ausubel *et al*. (1996). Cells from an overnight-grown culture on YPD (1.5 ml) were centrifuged at 1200x*g* and resuspended in 200 µl breaking buffer [2% (w/v) triton X-100; 1% (w/v) SDS; 100 mM NaCl; 10 mM tris·HCl, pH 8.0; 1 mM EDTA]. To break yeast cells, 0.3 g glass beads (Ø 425-600 µm; Sigma) and 200 µl 25:24:1 (v/v/v) phenol/chloroform/isoamyl alcohol (with buffered phenol, pH 8.0) were added and vigorous vortexing was applied for 2 min. The mixture was centrifuged at top speed in a microcentrifuge and 2 µl of top aqueous layer were used to transform *E. coli* as described previously. Plasmids were amplified by growth on LBamp of *E. coli* transformants (from one single colony) and isolated by the alkaline lysis method (Ausubel *et al.*, 1996; Sambrook *et al.*, 1989). Cells from the saturated culture (5 ml) were centrifuged at top speed in a microcentrifuge for 20 sec and resuspended in 100 µl GTE solution (50 mM glucose; 25 mM tris·HCl, pH 8.0; 10 mM EDTA). After 5 min incubation at room temperature, lysis was provoked by the addition of 200 µl NaOH/SDS solution [0.2 M NaOH; 1% (w/v) SDS] and incubation for 5 min on ice. Neutralization was done with 150 µl potassium acetate solution [5 M potassium acetate; 29.5% (v/v) glacial acetic acid; pH 4.8 adjusted with potassium hydroxide] with incubation 5 min on ice. Cell debris, denatured chromosomal DNA, and denatured proteins were removed by centrifugation at top speed in a microcentrifuge. Plasmid DNA

was precipitated with addition of 0.8 ml 95% (v/v) ethanol and incubation 2 min at room temperature. DNA was collected by centrifugation in a microcentrifuge at top speed, room temperature, and the pellet was washed with 70% (v/v) ethanol in order to remove contaminant salts. After drying of the pellet, plasmid was resuspended in ultrapure, nuclease-free water and analysed as describe before. Whenever necessary, RNA was removed, just before the precipitation step, by incubation with 5 µg/ml RNase, DNase-free (RNase, DNase-free from bovine pancreas; Roche) at 37ºC for 30 min. RNase was removed by extraction with 1 ml 25:24:1 (v/v/v) phenol/chloroform/isoamyl alcohol, recovery of aqueous phase and new extraction with 24:1 (v/v) chloroform/isoamyl alcohol. Isolated plasmids were used to transform *S. cerevisiae* strain YSH642 by electroporation and with the lithium acetate method. Yeast transformants were then checked for the presence of plasmid and the salt resistance phenotype by replica plating from YNBDALeu$^-$ master plates to YNBDA with 1.4 M NaCl and 50 mM glycerol.

Results

Osmotic sensitivity tests

The *gpd1gpd2* double mutant, being impaired in glycerol synthesis, cannot accumulate glycerol inside the cell in order to overcome the osmotic stress. Nevertheless, growth is achieved if the medium is supplemented with small amounts of glycerol (R. Ansell and S. Hohmann, personal communication), which could be explained by the activity of an active uptake for glycerol. So, the screening of the genomic library was based on this difference of osmosensitivity, which was studied for the laboratory conditions of the screening for designing convenient selective media. Strains disrupted in *GPD1* and *GPD2* show variation of osmosensitivity in synthetic and rich media depending on the presence of 50 mM glycerol (Figs. II.1 and II.2). The limit of tolerance of the recipient strain (*gpd1gpd2*) is 1M NaCl and the one of the parental strain (W303-1A) is 1.4 M NaCl in media with 50 mM glycerol. This difference allows the designing of the selective media for transformants obtained in the genomic library screening, assuming that high copy number of (the) gene(s) encoding the glycerol transporter, would restore the parental strain salt tolerance phenotype. Strains YSH294 (*fps1*) and YSH7.99.-3D (*gpd1gpd2fps1*) were included in these tests to evaluate a possible interference of *FPS1* in the screening. Simple disruption of *FPS1* increases salt tolerance as expected from a role involved in regulated glycerol efflux. In the case of the *fps1* mutation in a *gpd1gpd2* genetic background, osmotic sensitivity in synthetic medium is similar to *gpd1gpd2*, while in rich complex medium the supplementation with glycerol 50 mM does not improve growth under salt stress. The possibility of cloning *FPS1* as a suppressor of the osmotic sensitivity of *gpd1gpd2* in the presence of glycerol seems unlikely because if *FPS1* is present in a multicopy plasmid, more glycerol efflux would be possible with decreased salt tolerance. On the other hand, the increased expression of *FPS1* can cause increased amount of the Fps1p channel that are still regulated by salt stress in order to avoid glycerol efflux. In this case, an unaltered salt tolerance is expected to occur.

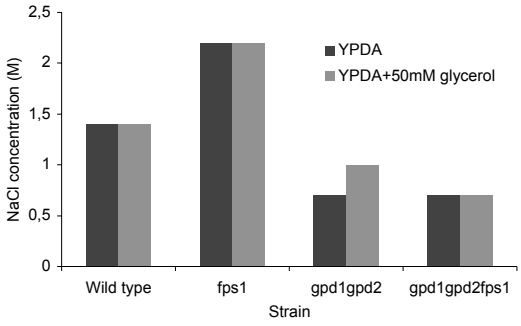

Figure II.1. Salt stress resistance of *S. cerevisiae* strains W303-1A, YSH294 (*fps1*), YSH642 (*gpd1gpd2*) and YSH 7.99.-3D (*gpd1gpd2fps1*) in rich complex medium with and without supplementation with 50 mM glycerol.

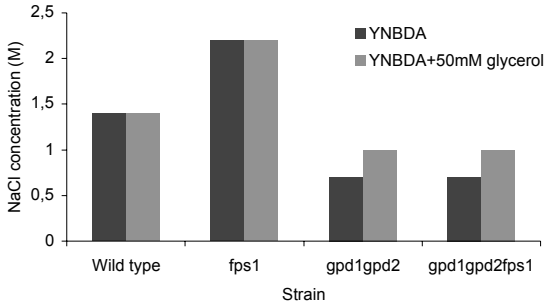

Figure II.2. Salt stress resistance of *S. cerevisiae* strains W303-1A, YSH294 (*fps1*), YSH642 (*gpd1gpd2*) and YSH 7.99.-3D (*gpd1gpd2fps1*) in synthetic medium with and without supplementation with 50 mM glycerol.

Genomic library screening

The first screen of transformants was done to confirm the presence of plasmid (Leu^+ phenotype) in order to quantify the transformants. Besides L-leucine, the selective synthetic medium lacked LD-tryptophan and uracil as well, with the purpose to eliminate revertants for the disruptions *gpd1::TRP1* and *gpd2::URA3*. A total of 50,000 clones were screened, corresponding to nearly 18 genome equivalents of *S. cerevisiae*. Transformants were then replica plated to salt resistance selective medium agar plates ($YNBDATrp^-Ura^-Leu^-$ + 1.4 M NaCl + 50 mM glycerol). From these, 502 clones were able to form colonies in this selective medium. Subsequently, the positive clones were restreaked to minimal medium ($YNBDATrp^-Ura^-Leu^-$) to confirm the presence of plasmid and the integrity of disruptions. Only 335 clones have grown in this medium, being frozen at -80ºC for further studies.

From frozen stocks, pre-inocula of 5 ml of YNBDATrp$^-$Ura$^-$Leu$^-$ medium were prepared, according to Materials and Methods, to confirm salt stress tolerance by drop tests of each positive clone with 1.4 M and 2 M NaCl with and without 50 mM glycerol. Growth was only observed in media with glycerol and more false positives were eliminated since only 31 clones were able to grow in the presence of 1.4 M NaCl and 19 with 2 M NaCl. Subsequently, the osmotic tolerance was determined using sorbitol and sucrose as stress agents, again, in media containing or not 50 mM glycerol. These results (not shown) allowed building the osmotic resistance profile for each clone which were grouped according to similar profiles of osmotic stress resistance increase with the addition of 50 mM glycerol (Table II.II).

Table II.II. Phenotypes of transformant cells of *S. cerevisiae*, strain YSH642 (*gpd1gpd2*), with a *S. cerevisiae* genomic library represented as increase in osmotic stress resistance to NaCl, sorbitol and sucrose by incorporation of 50 mM glycerol in the medium.

Clone representing each phenotype	Δ [NaCl] (M)	Δ [sorbitol] (M)	Δ [sucrose] (M)
229	0.4	0.3	>0.5
1560	>0.6	0.3	>0.5
2224	0.4	0.5	>0.5
2236	0.4	>0.5	>0.5
2311	>0.6	0.5	>0.5
2315	>0.6	0.3	0.5
2320	>0.6	0.8	0.5
2342	0.4	0.3	0.5
2380	0.4	>0.2	>0.5
2381	0.4	0.3	>0.5
2393	>0.6	0.5	0.5
2396	>0.6	>0.5	0
23124	>0.6	0.2	0.5
23133	0.4	0.2	0.5
23137	>0.6	0.5	0.5
23163	0.4	0.2	0.5
28123	0.4	0.5	0.5
YSH642	0.3	0.5	not determined
YSH642[YEp13]	0.3	0.5	not determined

Co-segregation and co-acquisition tests

The linkage between salt stress resistance phenotype and plasmid in each clone was tested by a co-segregation test and by a co-acquisition test. In the co-segregation test, cells were allowed to lose plasmids by growing in rich non-selective media and then plated in the same rich medium to obtain isolated colonies. By replica plating to plasmid-selective medium agar plates and to phenotype screening medium agar plates, the phenotype, if conferred by the plasmid, should co-segregate with the plasmid. Results obtained did not match with this assumption: cells of all clones exhibited salt stress resistance whether they possessed or had lost the plasmid. In order to confirm these results, cells from six clones chosen as representative and that had lost plasmid, were retransformed with copies of the lost plasmid using both the electroporation and the lithium acetate methods. The same procedure was applied to YSH642 cells transformed independently with these six plasmids. In all cases no acquired salt stress resistance phenotype was detected after transformation. One possibility could be the lack of genomic DNA insert in the isolated plasmids. However, the

sequence of the inserts from these six representative clones (kindly determined in the Carlsberg Laboratory, Copenhagen) corresponded to genomic DNA fragments as they closely matched sequences from the *Saccharomyces* Genome Database (Stanford University) by BLAST analysis (Table II.III).

Table II.III. Main features of six representative clones of the genomic library of *S. cerevisiae* isolated after screening for increased salt tolerance by transformation of cells of strain YSH642 (*gpd1gpd2*).

Clone	DNA fragment length (bp)	Chromosome	ORFs
2342	6040	XI	*YKL171w**, *MRPL38, YKL169c, KKQ8, MRP49*
2396	6234	VIII	*ERG11*, SOD2, YHR009c, RPL27**
2393	3701	VI	*YFR043c*, YFR044c, YFR045w*
23133	4135	XI	*FAS1*, YKL183w**
23137	5470	XIII	*SPT21*, YMR180c, YMR181c, RGM1, SSO2**
28123	5500	X	*YJL047c*, YJL048c, YJL049w, MTR4**

* incomplete ORF

Discussion

In studies concerning salt tolerance in *S. cerevisiae*, the glycerol transporter has never been mentioned as participating in this adaptation. Moreover, Lages and Lucas (1997) attributed a role of utilisation of glycerol as carbon and energy source under gluconeogenic conditions to this transporter, as no changes in uptake kinetic parameters and intracellular accumulation ratios were detected in cells grown in the presence and absence of sodium chloride (1 M) and in cells incubated in buffer containing 1 M NaCl. Nevertheless, the *gpd1gpd2* double mutant, unable to synthesise glycerol, presents an osmotic-sensitive phenotype which can be partially reverted with the addition of small amounts of glycerol to the medium (R. Ansell and S. Hohmann, personal communication) as can be seen in Figs. II.1 and II.2. This behaviour suggests the participation of an active uptake system for glycerol that could promote intracellular accumulation and, hence, overcome the lack of synthesis in order to compensate external salt stress. So, the strategy for the genomic library screening was based on high-copy cross-suppression because complementation is expected to occur not by the wild-type alleles of the genes mutated in the recipient strain (*GPD1* and *GPD2*), but by high-copy number of a different gene. It was assumed that if the glycerol transporter is present in a multicopy plasmid, the intracellular glycerol accumulation could be enough to completely revert the osmosensitive phenotype conferred by *gpd1gpd2* mutations. This means that in the screening, the clones containing the glycerol transporter would increase the osmoresistance from 1 M NaCl to 1.4 M NaCl in rich medium supplemented with 50 mM glycerol. However, the possibility of complementation with the wild-type alleles of *GPD1* and/or *GPD2* cannot be disregarded, and can only be analysed at molecular level.

In a previous work, Lages and Lucas (1997) have demonstrated that the glycerol active transport system is most probably under glucose repression,

being only detectable in cells grown in non-fermentable carbon sources. Hence, it would be likely that cloning the gene encoding this protein would be impossible because of the presence of glucose as carbon and energy source in all selective media. Nevertheless, the results presented in figures. II.1 and II.2, suggesting the activity of an active transport system for glycerol, were obtained in media with glucose using a strain impaired in glycerol synthesis (*gpd1gpd2*). Assuming that this effect is due to an active transport system for glycerol, this system would be active in glucose-grown cells provided the presence of glycerol and sodium chloride in the medium and the incapacity to synthesise glycerol. Another possibility is partial repression by glucose, decreasing the glycerol uptake to very low levels that would be sufficient for lack of detection of uptake in experiments with the radiolabelled substrate and that would not be high enough to avoid the effect observed with salt stress in agar plates.

Another glycerol transport system is known, being encoded by the *FPS1* gene (Van Aelst *et al.*, 1991; Luyten *et al.*, 1995) that could be caught in this screening. The participation of Fps1p in osmoregulation has been demonstrated in previous works (Luyten *et al.,* 1995; Tamás *et al.*, 1999; and Tamás *et al.,* 2000) and was tested with NaCl (Figs. II.1 and II.2) in order to exclude the possibility of cloning this gene in the screening. The *fps1* mutant strain (YSH294) showed, as expected, increased osmotolerance because of the lack of the channel, which contributes to higher intracellular retention of glycerol. Therefore, the possible presence of *FPS1* in a multicopy plasmid would not affect osmotolerance (in a *gpd1gpd2* genetic background) in opposition to the case of the presence of the active glycerol transporter gene in the multicopy plasmid. So, no interference of *FPS1* is expected although the triple mutant (YSH7.99.-3D: *gpd1 gpd2 fps1*) displayed an unexpected decreased osmotolerance in rich medium supplemented with 50 mM glycerol when compared with the YSH642 strain (Figs. II.1 and II.2). Presumably, these three mutations could lead to pleiotropic effects that, under mild osmotic stress such as rich complex medium with 0.7-1 M NaCl, the activity of an active uptake system for glycerol and subsequent intracellular concentration, could not be enough for survival in 1 M NaCl. On the other hand, when synthetic medium was used, with minimal contribution to osmotic stress, the increased osmotolerance is observed the same way as the one displayed by YSH642 strain (Figs. II.1 and II.2).

Although several clones have been isolated in the screening, we could not assign the phenotype to the plasmids by co-segregation and co-acquisition tests. The possibility of using an incomplete bank is likely because biases are always created due to the possible existence of zones with low number of restriction sites for *Sau*3AI and the low level of representativeness of areas around the centromeres and telomeres. However, the use of 18 genome equivalents for the screening aimed to circumvent this drawback, especially the case of areas with low number of sites for *Sau*3AI.

The isolated clones that turned out to be false positives can be produced by recombination induced by electroporation. Higgins and Strathern (1991) have reported that electroporation causes interchromosomal homologous mitotic recombination as measured by the formation of Trp+ and His+ recombinants in a diploid strain with mutant heteroalleles of the *trp1* and *his3* genes inserted in chromosome III. Although no interchromosomic homologous recombination is expected in haploid strains (like YSH642), the presence of genomic DNA in the

transforming plasmids in our screening can bring about homologous sequences to recombine with chromosomal DNA during electroporation.

Another common cause for unsuccessful cloning is lethality in *E. coli* and/or *S. cerevisiae*, considering the fact that the genomic library was constructed in a plasmid with pMB1 and 2µm replicons. Several genes involved in salt tolerance have been cloned by complementation with genomic libraries based in multicopy vectors such as *HAL1* (Gaxiola *et al.*, 1992) and *CNB1* (Mendoza *et al.*, 1994). In other works, libraries based in centromeric plasmids were used to clone *HAL2* (Gläser *et al.*, 1993), *GPD1* (Larsson *et al.*, 1993), and *GUT2* (Rønnow and Kielland-Brandt, 1993). In the case of *HAL2*, Gläser and co-workers (1993) have reported that increased dosage of 1-2 copies per cell in centromeric plasmid caused improved salt tolerance, but when dosage was increased to 10-40 copies per cell (in a 2µm-derived plasmid) no improvement of salt tolerance of transformants was observed. Although there was no way to address the problem of lethality, and despite the findings of Gläser and co-workers (1993), the option for a multicopy plasmid was based on the relative rarity of genes conferring toxicity/lethality in *E. coli* and *S. cerevisiae* and to physiological properties of the glycerol active transporter. These included the comparison with other yeasts for which glycerol active uptake had been characterised and its involvement in salt tolerance demonstrated. The values of active transport V_{max} were considerably high in glucose-grown cells, being for *Debaryomyces hansenii* 412 µmol.h^{-1}.g^{-1} dry weight (Lucas *et al.*, 1990), for *Zygosaccharomyces rouxii* 144 µmol.h^{-1}.g^{-1} dry weight (van Zyl *et al.*, 1990), for *Pichia sorbitophila* 1109.9 µmol.h^{-1}.g^{-1} dry weight (Lages and Lucas, 1995), and for *Pichia jadinii* 719 µmol.h^{-1}.g^{-1} dry weight (Lages *et al.*, 1999). Under the same growth conditions, using strain W303-1A of *S. cerevisiae*, no uptake was detected and no connection to salt stress response was observed (Lages and Lucas, 1997). Therefore, the use of a 2µm-based genomic library should increase the expression of the gene encoding the active glycerol transporter in order to get sufficient V_{max} to cause a physiological effect of salt tolerance through intracellular accumulation of glycerol.

In a parallel work (Holst *et al.*, 2000), aiming to clone the glycerol active transport gene in *S. cerevisiae*, an mTn-*lacZ/LEU2*-mutagenized plasmid library was used to create mutants by integration in homologous regions of the genome in the same strain used in this work. In this screen the genes *GUP1* and *GUP2* encoding the putative glycerol transporters were isolated. The difference between both approaches relied on the selection of the desired clones: while in transposon mutagenesis the selection was negative (lack of growth under salt stress) in a mutant strain, in the present approach the selection was positive (improved growth under salt stress) of a transformant. In our opinion, this difference was crucial since for systems that confer subtle phenotypes (such as the glycerol transport in *S. cerevisiae* in which V_{max} is very low when compared with glycerol transporters of other yeast species), it is more likely to detect lack of a function than an improvement. Consistently, Varela (1995) in a similar screening with a multicopy plasmid-based genomic library did not isolate genes conferring improved salt tolerance. So, new approaches should be considered in order to identify new genes involved in osmotic stress response in *S. cerevisiae* based not only in negative selection, but also with more discrimination capacity since the difference between osmotic stress and non-stressful osmotic pressure might be very tenuous.

Chapter III

Identification of putative glycerol transporter genes

Introduction

Early studies on glycerol transmembrane transport pointed to exclusively non-mediated mechanisms. Glycerol has been considered to enter *Saccharomyces cerevisiae* cells by simple diffusion, although with relatively low plasma membrane permeability that would allow retention of synthesised glycerol and, therefore, promote intracellular accumulation (Gancedo *et al.*, 1968). Subsequently, Blomberg and Adler (1989) have demonstrated that the intracellular accumulation of glycerol was due not only to increased synthesis, but also, to retention. By blocking glycerol synthesis induced by salt with cycloheximide that inhibits protein synthesis and, hence, further production of the key enzyme glycerol 3-phosphate dehydrogenase, the accumulation of glycerol was still observed in cells treated with 0.7 M NaCl.

Evidence of mediated transmembrane transport of glycerol was reported by Van Aelst and co-workers (1991) who cloned a gene, *FPS1*, by suppression of the growth defect of *fdp1* (also known as *CIF1* and *GGS1*) mutants in fermentable carbon sources, that was demonstrated to be a transport facilitator for glycerol (Luyten *et al.*, 1995; Sutherland *et al.*, 1997). Sutherland and co-workers (1997) presented evidence of a constitutively expressed glycerol transport system, with uptake kinetic parameters consistent with facilitated diffusion and that is unresponsive to metabolic inhibitors like CCCP. The absence of this type of transport in the *fps1* mutant and the homology of Fps1p to members of the MIP family of transport proteins, consisted good evidence that *FPS1* encodes the channel for glycerol facilitated diffusion. In addition, the *Escherichia coli* glycerol facilitator *glpF* (Sweet *et al.*, 1990), which is also a member of the MIP family of transport proteins, could partially complement *fps1* mutations (Luyten *et al.*, 1995).

Besides the pleiotropic effects originated by mutations in *FPS1* (Luyten *et al.*, 1995), which suggest additional functions, the capacity of glycerol uptake in a *fps1* mutant incubated under osmotic stress, pointed to the existence of more transport systems that, at least, would operate under these conditions. Lages and Lucas (1995) reported the uptake of radiolabelled glycerol in cell suspensions and the extracellular alcalinization of cell suspensions upon the addition of glycerol as physiological evidence of an active transport system for glycerol, of symport with protons type. Furthermore, this transport system promotes intracellular accumulation against gradient, which is prevented by the protonophore CCCP. A striking feature of this transport system is the regulation by glucose repression and gluconeogenic induction and the apparent non-involvement in osmotic stress. Molecular evidence for a gene encoding a glycerol active transport system came from Holst and co-workers (2000), who have performed transposon mutagenesis with a mTn-*lacZ/LEU2*-mutagenized library (Ross-Macdonald *et al.*, 1995) in a glycerol synthesis impaired strain (*gpd1gpd2*). Subsequently, they screened for mutants deficient in glycerol uptake and/or catabolism by the impairment of growth in media with glycerol as sole carbon and energy source and the inability to grow in YPD media supplemented with 1 M NaCl and 10 mM glycerol. One mutant with transposon insertion in the ORF *YGL084c* with predicted transmembrane domains and sharing homology with *YPL189w* was the most interesting candidate to code a transporter protein. Accordingly, the *ygl084c* disruption mutation in a wild-type background (fully able to synthesise glycerol), presented an osmosensitive phenotype that was not revertible with supplementation with 50 mM glycerol in glucose-based medium. On the other hand, *ypl189w* disruption mutation in a

wild-type background did not present any phenotype concerning glycerol utilisation and osmotic stress under the same growth conditions.

Materials and methods

Yeast strains, media and growth conditions

Saccharomyces cerevisiae strains used in this work are listed in table III.I. For glycerol uptake experiments and glycerol kinase enzymatic activities determinations, cells were grown in rich media [YP: 1% (w/v) yeast extract; 2% (w/v) peptone] with 2% (w/v) glucose (YPD), 2% (w/v) ethanol (YPE), or 2% (w/v) glycerol (YPG) as carbon and energy sources. Salt stress media were based on YPD with 1 M NaCl and 50 mM glycerol. Batch cultures were grown in Erlenmeyer flasks with culture/air proportion of 1/1 for growth when fermentable carbon and energy source was used (glucose). When non-fermentable carbon and energy source was used (ethanol or glycerol), the air/culture proportion was of 1/5. Growth was performed at 30ºC, 160 r.p.m. and was monitored spectrophotometrically at 600 nm.

Table III.I. Strains used in this work.

Strain	Genotype	Reference
W303-1A	*MAT**a** leu2-3, 112 ura3-1 trp1-1 his3-11, 15 ade2-1 can1-100*	Thomas and Rothstein (1989)
YSH392	Isogenic to W303-1A but *gpd1*::*TRP1*	Hohmann, S.
YSH6.141-4A	Isogenic to W303-1A but *gpd2*::*URA3*	Hohmann, S.
YSH642	Isogenic to W303-1A but *gpd1*::*TRP1 gpd2*::*URA3*	Hohmann, S.
	Isogenic to W303-1A but *gpp1*::*KanMX4*	Påhlman, A.-K. and Adler, L.
	Isogenic to W303-1A but *gpp2*::*KanMX4*	Påhlman, A.-K. and Adler, L.
	Isogenic to W303-1A but *gpp1*::*KanMX4 gpp2*::*HIS3MX6*	Påhlman, A.-K. and Adler, L.
BHY54	Isogenic to W303-1A but *gup1*::*His5*$^+$	Holst *et al.,* (2000)
CLy1	Isogenic to W303-1A but *gut1*	Holst *et al.,* (2000)
BHY40	Isogenic to W303-1A but *gut2*	Holst *et al*., (2000)
BHY65-1C	*MAT**a** ura3 leu2 trp1 his3 ade2 gut1 gup1*::*His5*$^+$	Holst *et al.,* (2000)
BHY51	Isogenic to W303-1A but *MAT*α *gpd1*::*LEU2 gut1*::*KanMX*	Holst *et al.,* (2000)
BHY56	Isogenic to W303-1A but *MAT*α *gpd1*::*LEU2 gup1*::*His5*$^+$	Holst *et al.,* (2000)
BHY22	Isogenic to W303-1A but MATα gpd1::TRP1 gpd2::URA3 gup1::Tn-LEU2	Holst *et al.,* (2000)
BHY61-1A	*MAT*α *ura3 leu2 trp1 his3 ade2 ygl084c*::*His5*$^+$ *fps1*::*HIS3*	Holst *et al.,* (2000)
CLy3	Isogenic to W303-1A but *ygl084c*::*His5*$^+$ *ypl189w*::*KanMX*	Holst *et al.,* (2000)
BHY67	*MAT**a** ura3 leu2 trp1 his3 ade2 gut1 ygl084c*::*His5*$^+$ *ypl189w*::*KanMX*	Holst *et al.,* (2000)
CLy4	Isogenic to W303-1A but *gpd1*::*LEU2 gup1*::*His5*$^+$ *gup2*::*KanMX*	Holst *et al.,* (2000)
BHY66	Isogenic to W303-1A but *MAT*α *gpd1*::*LEU2 gut1*::*KanMX ygl084c*::*His5*$^+$	Holst *et al*., (2000)

Glycerol transmembrane transport studies

Sample preparation

Cells were harvested at convenient growth phase by centrifugation at 7400x*g*, 4ºC, 2 min and washed twice with ice-cold deionised water. After washing, cells were resuspended in ice-cold deionised water to a final concentration of about 300 mg dry weight ml^{-1} and kept on ice. Dry weight was determined using 100 µl of cell suspension after drying at 80ºC for 24 hours.

For cells grown under salt stress, washing was done with ice-cold sodium chloride solution of the same concentration as the culture medium, as well as the final suspension of 300 mg dry weight ml^{-1}. Dry weight was determined by filtration of 100 µl of cell suspension through 0.2 µm membranes (Schleicher & Schuell), washed with the same amount of ice-cold deionised water, and dried at 80ºC for 24 hours.

Preparation of radioactive solutions

[^{14}C]glycerol (156 mCi/mmol, 250 µCi; Amersham) in 50% (v/v) ethanolic solution was used for uptake assays and all working solutions of radiolabelled glycerol were prepared according to Lages and Lucas (1997) and Holst *et al.* (2000). Since ethanol is known to affect plasma membranes (Leão and van Uden, 1984; Cartwright *et al.*, 1986; Petrov and Okorokov, 1990) and transport systems (van Uden, 1989), working solutions of radiolabelled glycerol were prepared in order to contain a maximum of 1% (v/v) ethanol. Firstly, ethanol was allowed to evaporate under vacuum until half of the original volume was reached (meaning that the ethanol has been evaporated). A stock solution was prepared with 200 µl [^{14}C]glycerol and 300 µl 250 mM unlabelled glycerol to obtain 150 mM final glycerol concentration with a specific activity of 400 dpm/nmol. From this solution, several working solutions were prepared by dilution, corresponding to a range of glycerol concentrations of 0.2-150 mM with the same specific activity. Solutions with different specific activities were also prepared by diluting radiolabelled glycerol with unlabelled glycerol in order to obtain less specific activity in more concentrated solutions. This way, a set of solutions ranging from 0.2 mM to 300 mM was obtained with specific activities ranging between 2800 dpm/nmol and 250 dpm/nmol respectivelly. All solutions prepared were stored at -20ºC in order to prevent proliferation of contaminants.

Initial uptake rates of radiolabelled glycerol

The experimental procedure for measuring initial uptake rates was in accordance with Lages and Lucas (1997) and Holst and co-workers (2000). Each assay was done with 6 mg dry weight of cells prepared as described previously, in buffer (100 mM tris·citrate, pH 5.0; with or without 1 M NaCl) in a water bath with control of temperature to 30ºC. After temperature stabilisation, 10 µl of radiolabelled glycerol was added (final reaction volume of 50 µl), and the reaction mixture was agitated. Reaction was stopped at convenient times by adding 5 ml ice-cold deionised water and separation of cells by vacuum filtration through a dampen membrane (Whatman GF/C). Cells were washed with 10 ml ice-cold deionised water and the membrane was then transferred to a vial

containing 5 ml liquid scintillation cocktail (OptiPhase HiSafe 2, Perkin Elmer). Glycerol uptake mediated by a transport system was determined with low concentrations of glycerol (0.2-8 mM) and short reaction times (up to 10 s). To determine non-mediated glycerol uptake, high concentrations of glycerol (10-60 mM) were used with longer reaction times (30-120 s). Specific activitiy of radioactive solutions was determined by measuring radioactivity of 5 µl of each radiolabelled glycerol solution in 5 ml liquid scintillation cocktail.

Kinetic parameters of active uptake following Michaelis-Menten saturation kinetics [K_m (mM) and V_{max} (µmol.h^{-}1.g^{-1}dry weight)] and diffusion constant from first order kinetic uptake [K_d (l^{-1}.h^{-1}.g^{-1}dry weight)] were calculated from Lineweaver-Burk or Eadie-Hofstee graphic representations. Statistic analysis of experimental data and calculation of kinetic parameters were done with a computer regression analysis program GraphPad Prism® (GraphPad Software). Results are mean values of, at least, three independent experiments.

Linearity between the amount of cells used in the assays and incorporation of radioactivity along time of exposure were evaluated, as done previously (Lages, 2000) in order to measure glycerol uptake within the linear range of the method. Reduction of radioactivity counts caused by sodium chloride was not observed. Adsorption of radiolabelled substrate to cell walls or plasma membranes was evaluated by inverting the order of addition of radiolabelled glycerol and water for stopping the reaction, in a control experiment. These values were subsequently used to correct values obtained in the assays.

Radiolabelled glycerol intracellular accumulation

[^{14}C]glycerol accumulation ratios were determined according to Lages and Lucas (1997). Yeast cells (30 mg dry weight) were incubated with 100 mM tris-citrate buffer (pH 5.0), with or without 1M NaCl, in total volume of 160 µl. The mixture was kept in a water bath at 30ºC, with magnetic agitation for 2 min. Reaction was started with 40 µl of radiolabelled glycerol and incubation prosecuted under the same conditions with withdrawal of 10 µl samples at convenient times. Cells of each sample withdrawn were immediately filtered through a glass microfibre membrane (GF/C, Whatman) and washed with 10 ml ice-cold deionised water. The membrane was then placed in a vial with 5 ml liquid scintillation cocktail and radioactivity was measured. The specific activity was determined directly using 10 µl of each radioactive solution in a vial with the liquid scintillation cocktail. Intracellular concentrations were calculated indexing radioactivity counts to intracellular volume according to the amount of cells present in the sample (as determined by Sutherland *et al.*, 1997 and Lages, 2000). The procedure was identical for cells incubated with or without 1 M NaCl. Intracellular and extracellular glycerol concentrations were plotted as in/out accumulation ratios over time. Results are mean values of, at least, three independent experiments.

The effect of the ionophore CCCP was assayed in order to determine the dependence of the glycerol uptake on ΔpH and $\Delta\Psi$. As an ionophore, CCCP has the property to abolish the pmf apparently by dissipating the transmembrane gradient of H^+ and, hence, inhibition of uptake of substrates by systems dependent on pmf and prevention of substrate accumulation driven by these transport systems. These assays were done by adding CCCP (10% (v/v) in ethanolic solution), to a final concentration of 50 µM, to the reaction mixture,

before addition of the radiolabelled substrate and after reaching in/out glycerol concentration equilibrium.

Glycerol-induced proton uptake

Proton movements across plasma membrane were determined using a pH electrode (Radiometer Copenhagen) to measure pH changes of cell suspensions. These were kept at 30ºC with magnetic stirring. Procedure used was according to Lucas and van Uden (1986), Loureiro-Dias (1988), and Lucas *et al.* (1990). Five ml of reaction mixture containing 1 mM tris-citrate (pH 5.0), 100-150 mg dry weight of yeast cells, and with or without 1 M NaCl, were incubated until stabilisation of temperature and pH. The cell suspension was adjusted to pH 5.0 with HCl and/or NaOH (at low concentrations) and a baseline was recorded. The substrate was added (to 10 mM final concentration) and extracellular pH variation over time was recorded, being the initial uptake rate of protons calculated from the deviation of the tangential slope of the initial alkalinization curve. Conversion of pH change to proton amount was done using a titrated HCl solution and results were expressed in µmol of protons $h^{-1}.g^{-1}$ dry weight.

Glycerol kinase assays

Cell-free extracts were prepared from cells harvested at convenient growth phase by centrifugation at 7400x*g*, 4ºC, 2 min and washed twice with ice-cold deionised water. Before the last centrifugation, cells were divided in aliquots (each corresponding to 125 ml of culture) and were frozen at -80ºC as a pellet by centrifugation at 4000x*g*, 4ºC, 5 min. Each aliquot was thawed on ice and resuspended in 1 ml ice-cold homogenisation buffer (50 mM MES; 1 mM EDTA; 150 mM NH_4SO_4; 1 mM DTT; pH 6.0 adjusted with NaOH) and was added 1.5 g glass beads (0.5 mm Ø). Cell disruption was done by vortexing 1 min five times with intercalated pauses of 1 min on ice. Cell debris were separated by centrifugation at 15000x*g*, 15 min, 4ºC and the supernatant was transferred to a new microcentrifuge tube. Protease activity was inhibited with a cocktail of proteases inhibitors (Complete™ Protease Inhibitor Cocktail Tablets; Roche) mixed in the cell-free extract, according to manufacturer's instructions and maintaining the temperature of the extract below 4ºC.

Glycerol kinase activity was determined in cell-free extracts indirectly, measuring the rate of reduction of NAD^+ with oxidation of glycerol 3-phosphate (catalysed by glycerol 3-phosphate dehydrogenase) produced in the first reaction of glycerol catabolism catalysed by glycerol kinase (Krakow and Wang, 1990). Reactions took place in spectrophotometric cuvettes in a spectrophotometer chamber with temperature regulated to 30ºC. The reaction mixture contained cell-free extract, assay buffer (200 mM glycine; 467 mM hydrazine; pH 9.0 adjusted with HCl), 10 mM NAD^+ (from yeast, Sigma), 10 mM glycerol, and 5 mM magnesium chloride. Absorbance at 340 nm was monitored until stabilisation and 20 µg/ml glycerol 3-phosphate dehydrogenase (*sn*-glycerol 3-phosphate:NAD^+ 2-oxireductase; EC 1.1.1.8; from rabbit muscle, Roche) was added. After recording for 2 min any possible ATP-independent NAD^+ reduction, 5 mM ATP (from bacterial source, Sigma) was added and variation in A_{340} was recorded for up to 10 min. Assays were repeated with at least three different amounts of cell-free extracts (10-20 µl) in order to check correlation between

protein extract and NAD^+ reduction. Enzyme activities were calculated based on the molar extinction coefficient of NAD^+/NADH (ε_{340} = 6300 $M^{-1}cm^{-1}$) and was expressed in activity units per milligram of total protein ($U.mg^{-1}$). Results are mean values of, at least, three independent experiments.

Total protein in cell-free extracts was determined with Folin-Ciocalteau reagent and with calibration with standards of bovine seric albumine. To 10^{-2} dilutions of extracts was added the same volume of solution A [0.01% (w/v) $CuSO_4.5H_2O$; 0.02% (w/v) sodium and potassium tartarate; 2% (w/v) Na_2CO_3; 0.1 M NaOH]. After homogenisation and incubation 10 min at room temperature, 1/4 volume of solution B [Folin-Ciocalteau phenol reagent (Merck) 1:1 diluted in deionised water] was added, mixed, and incubated 20 min. The absorbance at 750 nm was measured and protein concentration was calculated according to previous calibration with bovine seric albumine.

Results

The objective of this work was to identify the function of the proteins encoded by *YGL084c* and *YPL189w*. For *YGL084c*, direct involvement in glycerol catabolism and/or transport was known from phenotypes obtained with the insertion library mentioned above, but for *YPL189w* only homology at nucleotide and amino acid levels with *YGL084c* were available. The phenotypes of the *ygl084c* mutant strongly suggested a function related to glycerol uptake, hence the name *GUP1* for glycerol uptake, while for *YPL189w* only the sequence homologies at amino acid level of 54% identity and 69% similarity to Gup1p, suggested a related function.

Involvement of Gup1p in active transport of glycerol in ethanol-grown cells

To assign a function to Gup1p and Ypl189wp, glycerol uptake characterisation was performed in cell suspensions of mutant strains affected in genes known to be involved in glycerol metabolism (Table III.I). Additionally, some of these strains were further mutated in different combinations for *GUP1* and *YPL189w*. All these multiple mutants were constructed in W303-1A genetic background.

Strikingly, ethanol-grown cells of strain BHY54 (*gup1*) still presented a saturable uptake system as shown in Eadie-Hoffstee plots (Fig. III.1), with similar K_m value and a V_{max} decreased around 35% (Table III.II). The same results were obtained in double mutants with combinations of *gup1* with *fps1* (strain BHY61-1A) and *ypl189w* (strain CLy3) (Table III.II), indicating that the residual transport measured was apparently not due to either, the glycerol channel or the *GUP1* homologue. This was similar to the case of *E. coli*, in which glycerol kinase is the rate-limiting step in glycerol channel-mediated entry. Hence, the saturation kinetics measured was actually an artifact due to this phosphorylation step (Voegele *et al.*, 1993). Glycerol kinase is encoded by *GUT1* (Sprague and Cronan, 1977; Pavlik *et al.*, 1993) and under derepression conditions, it could phosphorylate glycerol entering cells by passive diffusion, leading to illusive increased influx. Therefore, the saturation kinetics measured could be due to this

intracellular radiolabel sequestering rather than to the activity of an active transport system. Since no proton uptake upon addition of glycerol was detected in cell suspensions of any of these three strains, the interference of glycerol kinase seemed to be more likely than an additional uptake system. Furthermore, the protonophore CCCP did not prevent this residual glycerol uptake (not shown).

Table III.II. Kinetic parameters of initial [^{14}C]glycerol uptake in cells grown in rich medium with ethanol as carbon and energy source (YPE) and harvested during exponential growth phase.

Strain	Relevant genotype	Km (mM)	Vmax (μmol h^{-1} g dw^{-1})	Kd (l h^{-1} g dw^{-1})
W303-1A	Wild type	1.1 ± 0.2	277 ± 26	0.010
BHY54	*gup1*	1.5 ± 0.5	181 ± 12	0.009
BHY61-1A	*gup1 fps1*	1.4 ± 0.6	137 ± 10	0.008
CLy3	*gup1 ypl189w*	1.6 ± 0.7	169 ± 17	0.008
CLy1	*gut1*	1.3 ± 0.4	205 ± 17	0.009
BHY51	*gut1 gpd1*	1.8 ± 0.6	248 ± 36	0.011
BHY66	*gut1 gpd1 gup1*	-	-	0.007
BHY67	*gut1 gup1 ypl189w*	-	-	0.007

- means absence of uptake

Glycerol kinase activity was measured in cell-free extracts of ethanol-grown cells. As expected, glycerol kinase activity levels were higher in W303-1A, YSH6.142-3D (*gpd1gpd2*), BHY54 (*gup1*) and CLy3 (*gup1ypl189w*) than in glucose-grown cells of wild-type W303-1A (Table III.III). Nevertheless, glycerol kinase activities did not demonstrate interference in experimental determination of glycerol uptake, therefore, new mutants were assayed for glycerol uptake: CLy1 (*gut1*), BHY51 (*gpd1gut1*), BHY66 (*gpd1gut1gup1*), and BHY67 (*gut1gup1ypl189w*) (Fig. III.1 and Table III.II). V_{max} values for glycerol uptake were decreased 26% in CLy1 and 11% in BHY51 as compared with W303-1A, clearly indicating the involvement of *GUT1* in uptake determinations. For BHY66 and BHY67, only simple diffusion could be measured (Fig. III.1 and Table III.II) therefore, these results suggest that the glycerol uptake measured with the radiolabelled substrate might be, actually, the sum of active uptake and simple diffusion with the contribution of kinetics of the first enzyme in glycerol catabolism, glycerol kinase.

Table III.III. Glycerol kinase activity measured in cells grown in rich medium with ethanol as carbon and energy source (YPE) and harvested during exponential growth phase.

Strain	Relevant genotype	Glycerol kinase activity on glucose-grown cells (mU mg $protein^{-1}$)	Glycerol kinase activity on ethanol-grown cells (mU mg $protein^{-1}$)
W303-1A	Wild type	3.2	66.1 ± 4.1
YSH642	*gpd1 gpd2*	5.4	55.8 ± 11.4
BHY54	*gup1*	3.4	43.1 ± 5.8
CLy3	*gup1 ypl189w*	4.5	48.9 ± 13.5

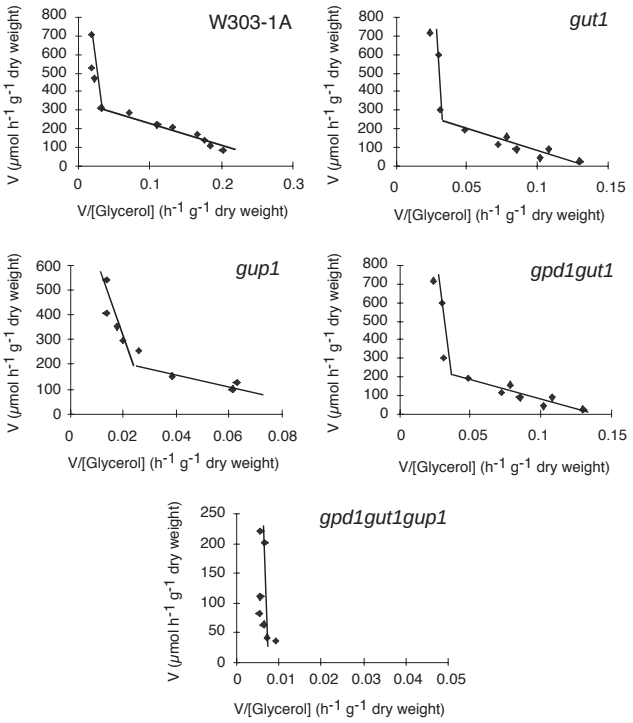

Figure III.1 Eadie-Hofstee plots of initial uptake rates of [^{14}C]glycerol by cells of *S. cerevisiae* strains W303-1A, CLy1 (*gut1*), BHY54 (*gup1*), BHY51 (*gpd1gut1*) and BHY66 (*gpd1gut1gup1*) harvested during exponential growth phase on 2% (w/v) ethanol.

To check if other enzymes of glycerol metabolism interfere with glycerol uptake determinations, different mutant strains disrupted in *GUT2*, *GPP1*, *GPP2*, *GPD1*, and *GPD2* as single or multiple mutants were assayed (Table III.IV). Only CLy1 (*gut1*) (Table III.II) and BHY40 (*gut2*) revealed a considerable decrease in V_{max} in relation to W303-1A, pointing to exclusive interference of catabolism in these determinations as predicted by results shown above.

Table III.IV. Kinetic parameters of initial [^{14}C]glycerol uptake in cells grown in rich medium with ethanol as carbon and energy source (YPE) and harvested during exponential growth phase.

Strain	Relevant genotype	Km (mM)	Vmax (μmol h^{-1} g dw^{-1})	Kd (l h^{-1} g dw^{-1})
W303-1A	Wild type	1.1 ± 0.2	277 ± 26	0.010 ± 0.001
CLy1	*gut1*	1.3 ± 0.4	205 ± 17	0.009 ± 0.001
BHY40	*gut2*	1.5 ± 0.7	212 ± 48	0.008 ± 0.002
	gpp1	1.6 ± 0.7	326 ± 62	0.011 ± 0.002
	gpp2	1.9 ± 0.2	318 ± 20	0.009 ± 0.001
	gpp1 gpp2	1.5 ± 0.3	250 ± 27	0.007 ± 0.001
YSH392	*gpd1*	1.3 ± 0.3	256 ± 27	0.008 ± 0.001
YSH6.141-4A	*gpd2*	1.2*	252*	0.009*
YSH642	*gpd1 gpd2*	1.6 ± 0.4	237 ± 30	0.013 ± 0.001

* - value obtained in only one assay

If Gup1p is actually an active transport system for glycerol, it should be capable of promoting intracellular accumulation against gradient. Accumulation

experiments performed in ethanol-grown cells with radiolabelled glycerol clearly suggested the activity of an active transport system in W303-1A cells (Fig. III.2). On the other hand, BHY54 strain (*gup1*) in similar experiments, presented lower levels of intracellular radioactivity and in a linear fashion over time, meaning that the entering glycerol was not actually being accumulated but metabolised into intermediate forms of glycerol catabolism. This is consistent with the effect of the protonophore CCCP when added after 30 min incubation, which did not prevent further radioactivity accumulation. On the other hand, in W303-1A cells, this drug just prevented a further increase in glycerol accumulation. The interference of glycerol catabolism in accumulation experiments was further confirmed in CLy1 (*gut1*) cells (Fig. III.2), with intracellular radioactivity reaching a *plateau* after 15 min incubation and an efflux of radioactivity upon addition of CCCP, which is explained by the existence of free intracellular glycerol. The effect of CCCP, when added from the beginning of incubation, corresponds to the prevention of intracellular accumulation of radioactivity consistently to the general long-term inhibitory effect as an uncoupler not only in plasma membrane but in mitochondria as well.

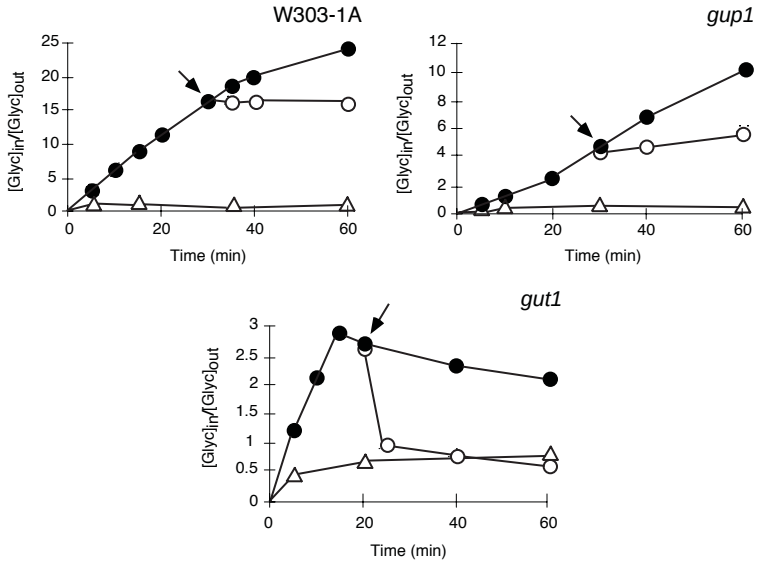

Figure III.2. Radiolabelled glycerol accumulation ratios in cells of *S. cerevisiae* strains W303-1A, BHY54 (*gup1*) and CLy1 (*gut1*), harvested during exponential growth phase on rich medium with ethanol as carbon and energy source (●). Influence of 50 μM CCCP on accumulation was determined by incorporation in the assay from the beginning of the experiments (Δ) and by addition at a given time point (arrow, ❍).

Involvement of Gup1p and Ypl189wp in active transport of glycerol in glucose-grown cells

Two characteristics are common to mutants isolated by Holst and co-workers (2000): deficiency in growth on glycerol and osmosensitivity in rich media with glucose as carbon and energy source supplemented with 1 M NaCl and 10 mM glycerol. The growth deficiency on glycerol is consistent with the proposed function of active glycerol transporter for *GUP1*. On the other hand, for *YPL189w*, experiments performed in ethanol-grown cells did not reveal any phenotype (Table III.II and III.III). Taking in consideration that the survey of

mutants obtained with the insertion library was made in medium containing salt and 15mM glycerol (Holst *et al*., 2000), the involvement of *GUP1* and *YPL189w* in salt stress on glucose-grown cells was investigated by uptake experiments in cells grown under these conditions and in glucose as carbon and energy source without salt stress or glycerol. Again, several mutant strains affected in genes of glycerol anabolism (*GPD1* and *GPD2*) and catabolism (*GUT1*) with mutations in *GUP1* and *YPL189w*, as single and multiple mutants, were tested in order to determine the function of *YPL189w*. The influence of glycerol metabolism in the activity of Gup1p and Ypl189wp was also studied.

In glucose-grown cells without salt stress, no transport activity was detected for glycerol in any mutant strain (Table III.V) in accordance to previous studies in wild-type *S. cerevisiae* (Lages and Lucas, 1997; Sutherland *et al.*, 1997). The same results were obtained with cells, grown on glucose with salt stress in the presence of 10 mM glycerol, of strains in which glycerol synthesis was not impaired (harbouring wild-type alleles of *GPD1* and *GPD2* simultaneously). Whenever *GPD1* alone or *GPD1* and *GPD2* were deleted, glycerol uptake was detected, being for YSH6.142-3D strain (*gpd1gpd2*) a V_{max} more than two times higher than for W303-1A in ethanol-grown cells. In strains with *gup1* deletion, together with *gpd1* (BHY56) or with *gpd1gpd2* (BHY22), glycerol uptake was detected, but for *gpd1gup1ypl189w* (CLy4) no uptake was detected. Furthermore, results with BHY56 and BHY22 clearly indicate that another transporter, besides Gup1p, is active and results with CLy4 strain strongly suggest that Ypl189wp might be that transport system. Consistently, all strains presenting glycerol uptake promoted extracellular alcalinization in cell suspensions upon glycerol addition (not shown), indicating, not only, that Ypl189wp might be a glycerol transport protein, but that either or both transport systems detected (*GUP1*-dependent and *YPL189w*-dependent) can correspond to the transport system characterised by Lages and Lucas (1997).

Table III.V. Estimation of the V_{max} (μmol h^{-1} g dry $weight^{-1}$) of glycerol uptake into salt-stressed or unstressed cells grown in rich media with glucose as carbon and energy source by measuring uptake of [^{14}C]glycerol at an extracellular concentration of 2 mM.

Strain	Relevant genotype	YPD	YPD + 1 M NaCl + 15 mM glycerol
W303-1A	Wild type	< 50	< 50
YSH642	*gpd1 gpd2*	< 50	630 ± 18
BHY54	*gup1*	< 50	< 50
CLy1	*gut1*	< 50	< 50
BHY65-1C	*gup1 gut1*	< 50	< 50
BHY56	*gpd1 gup1*	< 50	182 ± 0.5
CLy3	*gup1 ypl189w*	< 50	< 50
CLy4	*gpd1 gup1 ypl189w*	< 50	< 50
BHY22	*gpd1 gpd2 gup1*	< 50	379 ± 37

Similarly to ethanol-grown cells, glycerol uptake determined under salt stress could, in part, be due to derepression of glycerol kinase. Nevertheless, in cells grown strictly with glucose as carbon and energy sources, glycerol kinase activities were found to be very low compared to ethanol-grown cells (Sprague and Cronan, 1977). In addition, glycerol kinase derepression as a strategy to overcome salt stress seemed to be unlikely, because it would contribute to a decrease in intracellular glycerol concentration. Consistently, glycerol kinase activity measured in cell-free extracts in all strains presented in Table III.V and grown in glucose-based media with 1 M NaCl and 10 mM glycerol was less than

10% the activity measured in ethanol-grown cells of wild-type strain (data not shown).

Michaelis constants calculated evidenced a striking similarity of affinity for substrate of the transports systems analysed, being around 1.3 mM for *YPL189w*-mediated glycerol transport, similar to Km obtained for *GUP1*-dependent transport (Tables III.II and III.IV). In what concerns V_{max}, different values were obtained, according to the several mutation combinations. Interestingly, V_{max} of strain BHY22 (*gpd1gpd2gup1*) is considerably higher than BHY56 (*gpd1gup1*) (Table III.V), which points to a close dependence of *YPL189w*-dependent transport on glycerol synthesis impairment. In addition, the experimental detection of this transport system was dependent on salt stress and the presence of extracellular glycerol, suggesting different regulation mechanisms for *GUP1*-dependent and *YPL189w*-dependent glycerol transport.

In silico analysis of Gup1p and Gup2p

In similarity searches in public databases, including all non-redundant GenBank CDS translations, Protein Data Bank (PDB), Protein knowledgebase (SwissProt), Protein Information Resource (PIR), and Protein Research Foundation (PRF), using BLAST searching algorithm (Altschul *et al.*, 1990), four ORF were found to be homologous to the predicted amino acid sequence of Gup1p (Table III.VI). Remarkably, the homology to Gup1p of *Candida albicans* gene *IPF1764* translation product is the same as Ypl189wp in terms of score (identities and positives), although with lower statistical significance (E value). The overall homologies between these six ORF are highlighted in the best alignments performed with CLUSTALW program (Thompson *et al.*, 1994) (Fig. III.3). In these alignments several segments are highly conserved among these five proteins, which suggests similarity in function.

Table III.VI. Main features of the predicted proteins encoded by *GUP1*, *GUP2* and their closest homologues at amino acid level found by BLAST analysis: *SPAC24H6.o1c* from *Schizosaccharomyces pombe*, *IPF1764* from *Candida albicans* and *At1g57600* from *Arabidopsis thaliana*.

Predicted protein	GenBank accession number	Organism	BLAST expected value (E)	Similarity to Gup1p (identities)	Length (amino acids)	Predicted protein molecular mass	Predicted pI
Gup1p/Ygl084cp	NP_011431	*S. cerevisiae*	-	-	560	65 kd	9.50
Gup2p/Ypl189wp	NP_015135	*S. cerevisiae*	$1x10^{-161}$	54%	609	71 kd	9.59
Spac24H6.01cp	NP_592952	*Schizosaccharomyces pombe*	$1x10^{-86}$	41%	588	69 kd	9.34
Ipf1764p	-*	*Candida albicans*	$1x10^{-137}$	54%	584	69 kd	9.28
At1g57600p	NP_176073	*Arabidopsis thaliana*	$1x10^{-53}$	31%	533	63 kd	9.38

* - CandidaDB accession number: CA5274

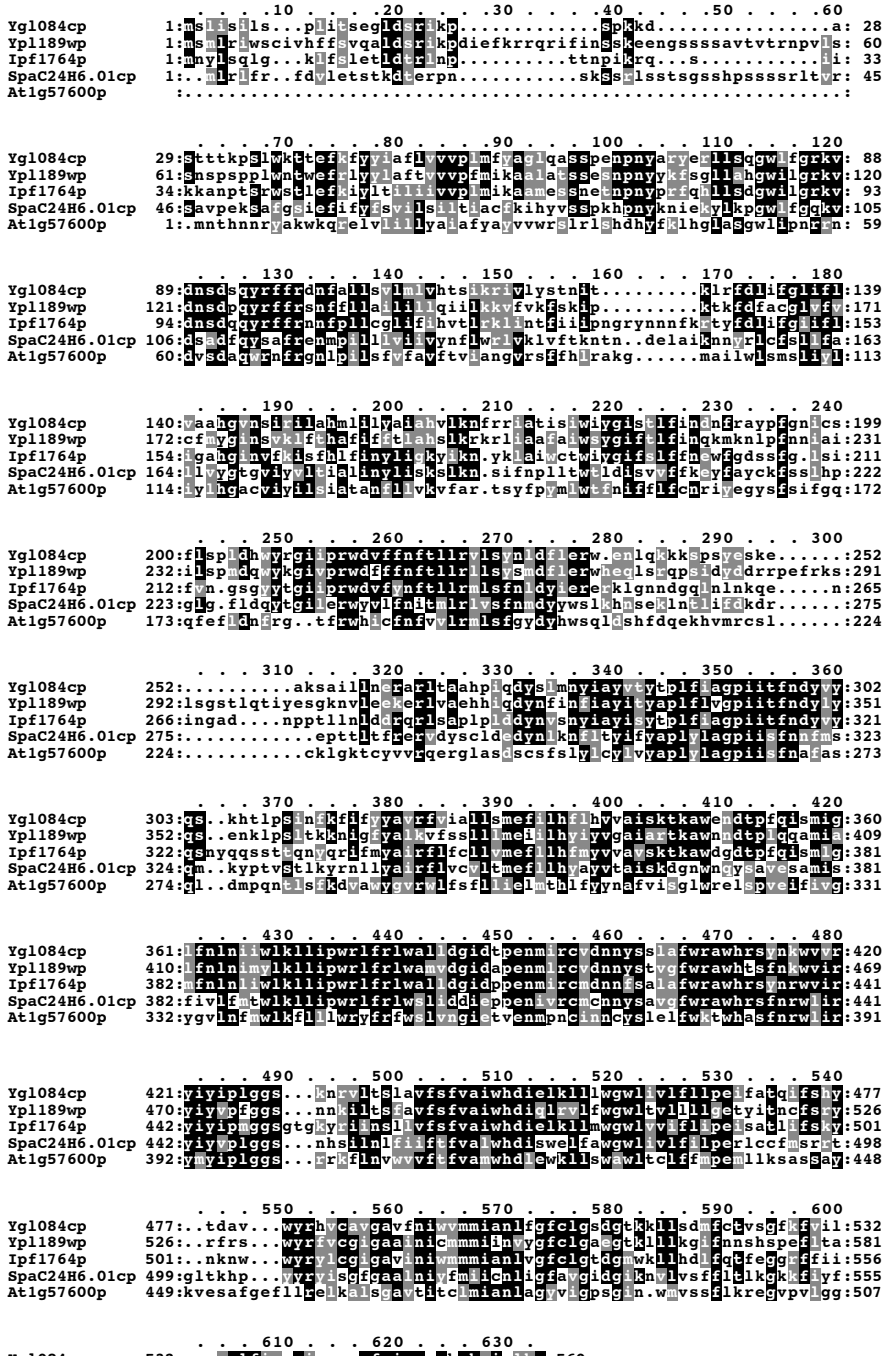

Figure III.3. Alignment, using the CLUSTALW program, of *GUP1* with *GUP2* and the closest homologues at amino acid level found by BLAST analysis in May 2003: *SPAC24H6.o1c* from *Schizosaccharomyces pombe*, *IPF1764* from *Candida albicans* and *At1g57600* from *Arabidopsis thaliana*. Conserved amino acids are shown in black boxes and similar amino acids are shown in grey boxes.

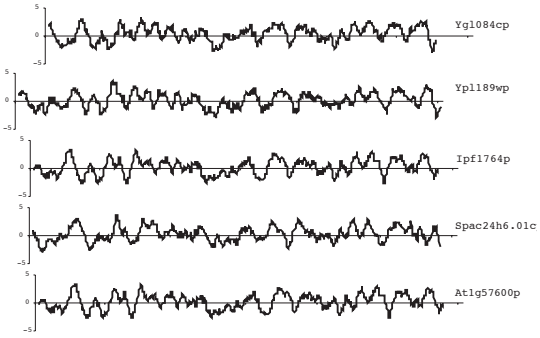

Figure III.4. Hydrophobicity profiles calculated according to Kyte and Doolittle (1982) of the predicted proteins encoded by *GUP1*, *GUP2* and their closest homologues at amino acid level found by BLAST analysis: *SPAC24H6.o1c* from *Schizosaccharomyces pombe*, *IPF1764* from *Candida albicans* and *At1g57600* from *Arabidopsis thaliana*.

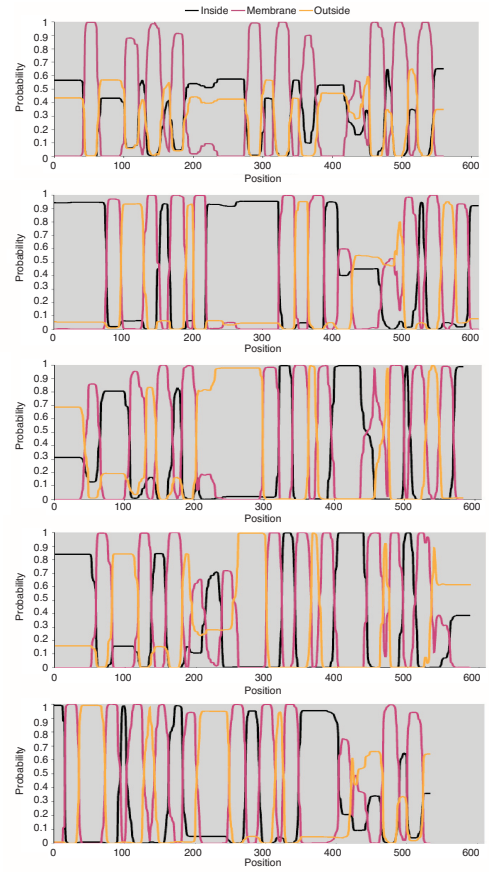

Figure III.5. Transmembrane domains prediction using the TMHMM helices prediction program (Sonnhammer *et al.*, 1998; Krogh *et al.*, 2001) of the predicted proteins encoded by *GUP1*, *GUP2* and their closest homologues at amino acid level found by BLAST analysis: *SPAC24H6.o1c* from *Schizosaccharomyces pombe*, *IPF1764* from *Candida albicans* and *At1g57600* from *Arabidopsis thaliana*.

The similarity is still found in hydrophobicity profiles calculated according to Kyte and Doolittle (1982) (Fig. III.4). Accordingly, all proteins present predicted

transmembrane domains obtained with TMHMM transmembrane helices prediction program (Sonnhammer *et al.*, 1998; Krogh *et al.*, 2001) (Fig. III.5). The proteins possess 9 to 11 predicted transmembrane domains arranged in four groups of closely placed transmembrane helices. The closest to the N-terminal end contains only one transmembrane helice being, the remaining groups composed of 2 to 4 transmembrane domains. The loop separating the two groups closer to the N-terminal end is usually shorter (32 to 39 amino acid residues) than the remaining loops, which are relatively large (37 to 118 amino acid residues). The N-terminal hydrophilic domain has 43-108 amino acid residues with intracellular position. The exceptions to these two characteristics are At1g57600p with a rather short N-terminal hydrophilic domain and Ipf1764p with extracellular localisation of the N-terminal domain. The hydrophilic C-terminal domain is usually very short, although Spac24h6.01cp presents an exceptionally long hydrophilic C-terminal. Hence, the overall arrangement of interconnecting hydrophilic loops and transmembrane domains are rather conserved, with few exceptions.

The localisation prediction of Gup1p and Gup2p according to PSORT II program (Nakai and Kanehisa, 1992) is consistent with the topological features, being with the highest probability in the plasma membrane (73.9% for Gup1p and 65.2% for Gup2p) and lesser probability, but not negligible, in the endoplasmic reticulum (21.7% for Gup1p and 34.8% for Gup2p). With much less probability (4.3%), Gup1p was predicted to be in mitochondria. The localisation in the plasma membrane suggests three essential functions: transmembrane transport, regulation of transport activity, and sensing of extracellular substrate or osmotic stress. The predicted phosphorylation sites obtained with the NetPhos 2.0 program (Blom *et al.*, 1999) in Gup1p (15 serine, 8 threonine, and 5 tyrosine residues) and Gup2p (21 serine, 3 threonine, and 7 tyrosine residues) are in agreement with any of these functions.

Discussion

Among the mutants obtained by transposon mutagenesis (Holst *et al.*, 2000), the one with an insertion in *YGL084c* was selected for further studies. The reason for this selection relied in the fact that this ORF was suggested to encode a multimembrane-spanning protein included in the major facilitator superfamily (Nelissen *et al.*, 1997). In addition, the phenotypes of reduced ability to grow on glycerol as carbon and energy source and the osmosensitivity in 1M NaCl with 10 mM glycerol, revealed during the isolation, indicated that *YGL084c* could code the permease responsible for the active uptake of glycerol and hence named *GUP1*. These phenotypes were detected in a *gpd1gpd2* genetic background, leaving to the supplemented external glycerol the only source of the compatible solute to overcome osmotic stress. In this work, several yeast strains affected in glycerol metabolism were analysed and new evidence was obtained sustaining the role of glycerol active carrier for Gup1p. In ethanol-grown cells, uptake assays (Fig. III.1 and Table III.II) and in/out accumulation ratios of glycerol (Fig. III.2) clearly demonstrated an involvement in glycerol uptake and salt stress response. In strains bearing simultaneously *gup1* and *gut1* disruption mutations no active uptake was detected. In mutants affected in *GUP1* or *GUT1*, partial abolishment of glycerol uptake was observed, indicating that the phosphorylation of glycerol by glycerol kinase encoded by *GUT1* contributes to the experimentally determined glycerol uptake. This was coherent with the differences between W303-1A and CLy1 (*gut1*) strains concerning glycerol

accumulation ratios. While strain W303-1A presents an in/out ratio of 25, CLy1 presents only an in/out of 3 (Fig. III.2), showing the participation of glycerol catabolism in measured glycerol accumulation. Consistently, the efflux caused by CCCP seen in W303-1A and CLy1 cells corresponded to the actual free intracellular glycerol. Assuming a transport mechanism of symport with protons, as has been demonstrated to exist in *S. cerevisiae* (Lages and Lucas, 1997; Sutherland *et al.*, 1997), the lack of detection of proton movements in cell suspensions upon addition of glycerol in strains affected in *GUP1* (BHY54, BHY61-1A, and CLy3), together with the partial inhibition of the measured glycerol uptake by CCCP are still concordant with this assumption.

In a wild-type background, *gup1* mutation causes a phenotype of slow growth in media with glycerol as carbon and energy source and in rich media with glucose as carbon and energy source with 1 M NaCl with 10 mM glycerol (Holst *et al.*, 2000). These results indicate that even when glycerol synthesis is not affected, *GUP1* is involved in glycerol utilisation and in intracellular retention by uptake of leaking glycerol under salt stress. Nevertheless, in *gup1* mutants grown on glucose and salt and on glycerol active uptake has never been detected with the radiolabelled substrate. One explanation for this could be an activity below the detection level of the experimental approach, signifying that the actual function of *GUP1*, besides uptake of glycerol to utilise as carbon and energy source, could be fine tuning adjustment of intracellular glycerol concentration under osmotic stress. This is coherent with low levels of V_{max} measured experimentally in the wild type strain (277 $\mu mol.h^{-1}.g.dw^{-1}$) when compared with V_{max} of other proton/symport systems from the same strain of *S. cerevisiae* like, for instance, acetate (4896 $\mu mol.h^{-1}.g.dw^{-1}$) and lactate (792 $\mu mol.h^{-1}.g.dw^{-1}$) (Paiva *et al.*, 1999). Additionally, the low velocity of glycerol disappearance of the medium, reported by Lages and Lucas (1997), when cells grow in rich medium with glycerol as carbon and energy source and the phenotype of slower growth in glycerol of the mutant *gup1* could be consequences of this weak activity. Consistently, the codon bias index calculated for both genes is extremely low (according to the *Saccharomyces* Genome Database from Stanford University: 0.135 for *GUP1* and 0.000 for *GUP2*), indicating a small proportion of optimal codons in these genes, leading, presumably, to low expression.

In ethanol-grown cells, no relation to glycerol uptake could be attributed to *YPL189w* because the deletion of this ORF did not modify the kinetic parameters of glycerol uptake (Table III.II). In addition, Holst and co-workers (2000) reported the absence of phenotype in terms of growth and osmosensitivity by deletion of *YPL189w* either in wild-type genetic background (strain CLy5), and *gup1* genetic background (strain CLy3). Accordingly, a genomic copy of *YPL189w*, in either centromere-based or multicopy vector, did not complement *gup1* deletion in media with glycerol as carbon and energy source and in salt stress media (YPD + 1 M NaCl) (Holst *et al*., 2000). Yet, cells unable to synthesise glycerol (*gpd1gpd2* genetic background), when grown on glucose with 1 M NaCl and 15 mM glycerol, exhibited an uptake more than two times the activity measured in wild-type cells grown in ethanol (Tables III.II and III.V). This activity was only partially abolished with deletion of *GUP1* (strain BHY22), being completely abolished when *GUP2* was also deleted (strain CLy4). As expected, under these conditions, glycerol kinase did not influence glycerol uptake, as demonstrated in these two strains by the very low level of enzymatic activity of glycerol kinase measured in cell-free extracts (data not shown). So, besides the close dependence of *YPL189w*-dependent transport activity on the capacity for glycerol synthesis as shown by strains *gpd1gup1* (strain BHY56) and *gup1* (strain BHY22) (Table III.V), Ypl189wp seems to actively transport glycerol as

well with an affinity constant similar to the Gup1p-driven uptake. Remarkably, the deletion mutants *gup1* (strain BHY22) and *gpd1gup1gup2* (strain CLy4) also allow to indirectly detect glycerol uptake dependent of *GUP1*. Again, the possibility of activity of Gup1p in glucose-grown cells mentioned above is supported with these results.

In accumulation experiments, the capacity of promoting intracellular accumulation against a concentration gradient is measured. As mentioned before, the comparison of the accumulation ratios as well as the efflux of free radiolabel after accumulation in the strains tested (Fig. II.2), demonstrate some participation of the first step of glycerol catabolism in the uptake measured with the radiolabelled substrate. Only with *gut1* (strain CLy1) the actual free glycerol intracellular accumulation could be determined due to the impairment in the first reaction of glycerol consumption. This was much less than the wild-type strain in cells grown with ethanol as carbon and energy source, which clearly indicates that, at least under derepressing conditions, transport and phosphorylation of glycerol are physiologically related. Hence, a direct interaction at molecular level between Gut1p and Gup1p, directly or indirectly, is plausible as has been demonstrated in *E. coli* for the case of the phosphotransferase system (Voegele *et al.,* 1993). Interestingly, glycerol kinase activities determined match with glycerol uptake V_{max} in terms of the carbon source: higher values for ethanol-grown and undetectable or very low levels for glucose-grown cells. Actually, *GUT1* expression was demonstrated to be under the control of the carbon source (Grauslund *et al.,* 1999), and this concurrence of activities suggests involvement in the same physiological mechanisms, although this is unexpected to occur under salt stress where glycerol intracellular accumulation is one of the mechanisms of osmotic stress response.

Diffusion constants in ethanol-grown cells were invariant regardless of the mutant strain tested (Tables III.II and III.IV). Sutherland and co-workers (1997) found differences on the lipid composition of the plasma membrane between wild-type and the *fps1* mutant, with different diffusion constants. These authors reported that *fps1* mutants presented lower apparent membrane permeability than the wild-type, which could be an adaptation to osmotic stress leading to intracellular glycerol retention. On the other hand, *GUP1* deletion causes an increase in triglyceride synthesis and a simultaneous decrease in phospholipid synthesis (Oelkers *et al.,* 2000). Therefore, it is conceivable that this interference in membrane lipid composition by *GUP1* should modify plasma membrane permeability towards glycerol. However, neither *GUP1* nor *GUP2*, changed permeability (diffusion constant, Kd) of plasma membrane to glycerol. This issue, which remains to be elucidated, is especially relevant in osmotic stress when a decrease in permeability of plasma membrane could contribute to favour glycerol intracellular retention.

The uptake of glycerol by Gup1p requires a localisation in the plasma membrane, spanning both layers of phospholipids in order to expose parts of the molecule to the outer and inner sides of the membrane. Therefore it is important to determine the localisation of a given putative carrier protein in order to contribute to the knowledge of its function as a carrier. The fact that a protein is localised in a membrane does not necessarily mean that it is a carrier protein, although integral membrane proteins may act as sensors as well. On the other hand, the membrane localisation prediction software used (PSORT II), is based on amino acid sequence comparisons with a knowledge base consisting of a compilation of known sorting signals for each subcellular localisation (Nakai and Kanehisa, 1992) and is given as a probability. Holst and co-workers (2000) using a haemagglutinin-tagged version of Gup1p (Gup1p-HA) under the *GUP1*

promoter control in a centromere-based plasmid have co-localised Gup1p with the plasma membrane H^+-ATPase after separation of the membrane fraction in sucrose density gradient. This result matches with the localisation predicted by PSORT II program (Nakai and Kanehisa, 1992) for Gup1p giving highest probability for plasma membrane localisation. However, this program also predicted localisation at the endoplasmic reticulum membrane and the mitochondrial membrane, although with much less probability, but with striking accordance with the experiment of Holst and co-workers (2000) in which some degree of co-localisation with endoplasmic reticulum NADPH-cytochrome oxidase and with mitochondrial cytochrome oxidase was found (Holst *et al.*, 2000).

Sequence similarity between *GUP1* and *GUP2*, at both nucleotide (not shown) and amino acid level (Fig. III.3), is maintained in hydrophobicity profile (Fig. III.4) and number and arrangement of transmembrane spanning regions (Fig. III.5). Accordingly, Nelissen and co-workers (1997) have classified *YGL084c* (*GUP1*) and *YPL189w* (*GUP2*) as members of an unknown family belonging to the major facilitator superfamily. In addition, the physiological data concerning *GUP2*, in particular the glycerol uptake measured in strain BHY56 (*gpd1gup1*) when cells growing on glucose are subjected to salt stress, allowed to attribute to *GUP2* a function of permease for glycerol.

BLAST searches in public databases unveiled, besides *YPL189w* (*GUP2*), three more ORF's (Table III.VI) with high sequence homology with *GUP1*: *IPF1764* from *Candida albicans*, *SPAC24H6.01c* from *Schizosaccharomyces pombe*, and *At1g57600* from *Arabidopsis thaliana*. In *C. albicans* and *A. thaliana*, no studies have been made so far concerning the existence of an active uptake for glycerol. On the other hand, *S. pombe* was included in a survey for active glycerol uptake in halotolerant yeasts (Lages *et al.,* 1999) in which, surprisingly, no active uptake of radiolabelled glycerol was detected neither in cells grown on glucose nor on glycerol. This could mean that *SPAC24H6.01c* is not a transporter for glycerol or special induction conditions are necessary to uncover the transport activity. With the increase in genome-wide research, more genome sequences from many organisms, including yeasts, are becoming available. This way, *GUP1*/*GUP2* homologues might be revealed starting simply by sequence homology search in many halotolerant yeast species that have been demonstrated to possess glycerol active uptake systems (Lages *et al*., 1999), including those for which this transport system was already charaterised, like *Debaryomyces hansenii* (Lucas *et al*., 1990) and *Pichia sorbitophila* (Lages and Lucas, 1995). A function of glycerol carrier for *IPF1764* and *SPAC24H6.01c* is suggested by the close homologies shared with *GUP1* and *GUP2*, although this function has not been conclusively demonstrated for *GUP1* and *GUP2*.

The phenotypes associated with *GUP1* and *GUP2* deletions revealed in this study clearly indicate that the expression of these two genes is differently regulated. In fact, (i) only Gup1p is active in ethanol-grown cells; (ii) *GUP1* is essential for growth on glycerol as carbon and energy sources; (iii) *GUP1* contributes to recovery from salt stress media with glucose as carbon and energy sources; (iv) Gup2p is only active in salt-stressed cells on glucose when glycerol synthesis is impaired; and (v) deletion of *GUP2* does not affect growth in any carbon source, then it seems that *GUP1* and *GUP2* could function in different physiological mechanisms. Such discrimination of regulation between proteins involved in the same metabolic step in glycerol metabolism is not new. It is the case of *GPD1* and *GPD2* encoding two isoenzymes of glycerol 3-phosphate dehydrogenase and *GPP1*/*RHR2* and *GPP2*/*HOR2* encoding two isoenzymes of glycerol 3-phosphate phosphatase being, these four enzymes

involved in glycerol synthesis pathway. Glycerol synthesis driven by osmotic stress involves essentially increased expression of *GPD1* (Eriksson *et al.,* 1995; Ansell *et al.,* 1997; Rep *et al.,* 1999a; Eriksson *et al.,* 2000) and *GPP2*/*HOR2* (Norbeck *et al.,* 1996), while *GPD2* is mainly involved in redox regulation (Ansell *et al*., 1997) and *GPP1*/*RHR2*, although involved in osmoadaptation as well, is involved in adaptation to anaerobic conditions (Påhlman *et al.,* 2001a).

The function of *GUP1* and *GUP2*, suggested by the evidence presented, is the transmembrane transport of glycerol by a mechanism of symport with protons. However, data are not fully conclusive regarding an exclusive role in transmembrane transport for glycerol, since other functions may be attributable to membrane proteins, in particular sensing or regulating transport. Recently, *YGL084c* (*GUP1*) and *YPL189w* (*GUP2*) were included in the so-called MBOAT (for membrane-bound *O*-acyl transferases) superfamily (Hofmann, 2000), as they share sequence homology to diverse membrane-associated acyltransferases. Members of this superfamily include the human *ACAT1* and *ACAT2* genes involved in fatty acid transfer to cholesterol and the yeast *ARE1* and *ARE2* genes, encoding enzymes catalysing the transfer of fatty acids to sterols. Moreover, all the biochemically-characterised members of this superfamily encode enzymes that transfer organic acids to hydroxyl groups of compounds placed in the membrane. Likewise, in an independent work, Oelkers and co-workers (2000) identified *YGL084c* (*GUP1*) and *YPL189w* (*GUP2*) as homologues of the mammalian *ACAT1* and *ACAT2* and the yeast *ARE1* and *ARE2* genes. In biochemical characterisation of mutants in these four yeast genes, they were able to find a temperature-sensitive phenotype in *ygl084c* and, again in this strain, a modification in lipid synthesis: increase of triglyceride and decrease of phospholipid. It is noteworthy that the temperature-sensitive phenotype of *gup1* mutant is shared by *gpd1gpd2* and *gpp1gpp2* mutants and that cells accumulate glycerol under heat stress (Siderius *et al.,* 2000). These new features expand the possibilities for functions of *GUP1* and *GUP2* that could act at regulatory level, controlling intracellular levels of glycerol in response to osmotic and heat stresses and nutrient availability. Nevertheless, these putative new functions are not incompatible to a glycerol uptake function for *GUP1* and *GUP2* as is strongly suggested by the evidence presented here.

Expression studies of the putative glycerol active transport genes of *Saccharomyces cerevisiae*, *GUP1* and *GUP2*

Introduction

In yeasts, active uptake systems for glycerol have been described previously (Lages *et al*., 1999), although only a minor proportion of them were characterised in detail (Adler *et al.,* 1985; Lucas *et al*., 1990; van Zyl *et al*., 1990; van Zyl *et al.,* 1993; Lages and Lucas, 1994; Oliveira *et al*., 1996; Silva-Graça and Lucas, 2002). The predicted function of these transport systems has been connected with the utilisation of glycerol as carbon and energy source, since all yeast species presenting such active uptake systems were able to utilise glycerol for growth. On the other hand, as glycerol is found among yeasts as the main compatible solute, an active uptake of the compatible solute could contribute to intracellular retention and accumulation. Thus, the obvious functions that have been attributed to these transport systems were (i) the first step in glycerol catabolism and (ii) the participation in intracellular glycerol accumulation, as compatible solute, to overcome osmotic stress.

Evidence suggesting a connection between active glycerol uptake and halotolerance has been reported for yeasts presenting high salt-tolerance like *Candida halophila*, *Candida nodaensis*, *Stephanoascus ciferrii*, *Wingea robertsiae,* and *Pichia sorbitophila* (Lages *et al*., 1999). All these yeasts presented constitutive glycerol uptake and proton uptake upon addition of glycerol, suggesting a mechanism of H^+/glycerol symport, and intracellular accumulation of glycerol independent of the presence of salt. Accordingly, Lages and Lucas (1995) demonstrated that the accumulated glycerol in *P. sorbitophila* cells was not being used as carbon source in the presence of glucose. In addition, Oliveira and co-workers (1996) isolated mutants of *P. sorbitophila* defective in H^+/glycerol symport based exclusively in increased osmotic sensitivity after random mutagenesis. In the case of *C. halophila*, the glycerol active uptake was suggested to be one of the mechanisms involved in response to salt stress, but taking in consideration its extreme halotolerance, other physiological mechanisms should be as important as glycerol uptake for intracellular retention of the compatible solute (Silva-Graça and Lucas, 2002).

Another group of halotolerant yeasts presented salt dependence of glycerol intracellular accumulation and proton uptake upon addition of glycerol, besides the common characteristic of the absence of repression by glucose over the glycerol transport. The uptake mechanism of the most studied yeast of this group, *Debaryomyces hansenii*, was proposed to be sodium-glycerol co-transport (Lucas *et al*., 1990), since glycerol-elicited proton uptake was only detectable in the presence of external sodium chloride. In *Zygosaccharomyces rouxii*, the driving force for glycerol uptake is apparently coupled to external concentration of NaCl as well (van Zyl *et al*., 1990). However, in both yeasts, the intracellular accumulation is done, in a subsequent adaptation phase, by biosynthesis (van Zyl *et al*., 1993; Neves *et al.,* 1997).

Intracellular accumulation of any metabolite can be achieved by active uptake systems, increased biosynthesis, intracellular retention, or by all these mechanisms. In the case of glycerol, Lages and co-workers (1999) have found that yeast species that presented proton uptake elicited by glycerol presented intracellular accumulation of this compound. Moreover, specific growth rates of

yeasts possessing constitutive salt-independent active glycerol transport are not markedly lower for growth in glycerol than in glucose as carbon and energy source. Therefore, constitutive active uptake systems were demonstrated to be involved not only in intracellular accumulation as response to salt stress but in efficient assimilation of glycerol as well.

In contrast of constitutive uptake systems, repressible glycerol active uptake seems to have a role exclusively connected to glycerol catabolism, as is the case of *S. cerevisiae* (Lages and Lucas, 1997; Lages *et al.,* 1999). This is strongly suggested by the carbon source-dependence of expression and the lower salt-tolerance of these yeasts when compared to those presenting constitutive glycerol uptake systems. The putative genes encoding the glycerol transporter in *S. cerevisiae*, *GUP1* and *GUP2*, were cloned, sequenced, and characterised (Holst *et al*., 2000). A remarkable feature of *GUP1* is its involvement in glycerol-mediated recovery from salt stress, besides the expected participation in glycerol assimilation. Considering that glycerol uptake has been referred to be under glucose repression (Lages and Lucas, 1997) and that the salt stress response involvement was detected in media with glucose as carbon and energy source (Holst *et al.,* 2000), *GUP1* might be regulated for osmotic stress response as well. For *GUP2*, instead, no phenotype was detected in a wild-type genetic background. However, a strong glycerol uptake activity was measured in a *gpd1gpd2gup1* genetic background under salt stress. This could mean that *GUP2* might act as an alternative mechanism for glycerol intracellular accumulation, provided the inability to synthesise this compound.

Yeasts that lack salt-activated uptake systems for glycerol have been reported to accumulate glycerol through an increase in biosynthesis (Brown, 1978). *S. cerevisiae* has been included in this group of yeasts since the active uptake system characterised physiologically is unresponsive to salt and repressed by glucose (Lages and Lucas, 1997). In addition, high levels of glycerol are produced allowing restoring cytoplasmic redox balance under anaerobic conditions (van Dijken and Scheffers, 1986) without the need of induction by osmotic stress. In this case, upregulation of *GPD1* (Albertyn *et al*., 1994a; Ansell *et al*., 1997) and *GPP2*/*HOR2* (Påhlman *et al.,* 2001a) provide increased capacity of glycerol synthesis. The hypothesis that intracellular accumulation of glycerol in *S. cerevisiae*, under salt stress, is done at increased synthesis expense is still consistent with the activity of the glycerol channel Fps1p. This protein was reported to be responsible for glycerol facilitated diffusion (Sutherland *et al*., 1997) and, hence, being unable to promote intracellular accumulation. The same authors reported the involvement of *FPS1* in lipid biosynthesis and considered this an indication that *FPS1* affects plasma membrane permeability, contributing to glycerol retention. In addition, Luyten and co-workers (1995) presented evidence of *FPS1* involvement in increased glycerol synthesis by a mechanism that is still not understood. More recently, Tamás and co-workers (1999) have reported that Fps1p is involved in glycerol efflux and its expression is not controlled by osmotic stress. These observations suggest an adaptation to changes of osmolarity through opening/closure of the channel so that intracellular levels of glycerol may be regulated. In this mode of action of Fps1p, a N-terminal domain close to the first transmembrane domain is involved as the truncated mutants in this region present constitutive glycerol efflux and increased osmotic sensitivity (Tamás *et al*., 1999).

Results obtained by Holst and co-workers (2000) seem to contradict the proposed strategy for osmoregulation in *S. cerevisiae*, since *GUP1* contributes to salt stress tolerance and is active under repression conditions. This evidence is subtle in terms of phenotype and the question that remains to answer is at what extent the phenotypes revealed by the *gup1* mutant are actually part of an osmotic stress response. As previously mentioned, one possibility could be fine-tuning of intracellular levels of glycerol for which the main contributor would be biosynthesis and, subsequently, the active uptake system would keep convenient intracellular levels by transporting glycerol that had been leaked out of the cell. So, expression analysis of *GUP1* and *GUP2* constitute an approach to understand the physiological function of these genes.

Materials and Methods

Yeast strains, media and growth conditions

Saccharomyces cerevisiae strains W303-1A (*MAT***a** *leu2-3, 112 ura3-1 trp1-1 his3-11, 15 ade2-1 can1-100*) (Thomas and Rothstein, 1989) and YSH642 (isogenic to W303-1A but *gpd1::TRP1 gpd2::URA3*) were used for expression studies of *GUP1* and *GUP2* genes. Cells were grown in rich medium [1% (w/v) yeast extract and 2% (w/v) peptone] containing 2% (w/v) glucose (YPD), 2% (w/v) ethanol (YPE), or 2% (w/v) glycerol (YPG) as carbon and energy source. Salt stress media were prepared with YPD supplemented with 1M NaCl with or without further supplementation of 50 mM glycerol. Growth was performed at 30ºC in Erlenmeyer flasks with medium/air relation of 1/1 for glucose as carbon and energy source and 1/5 for non-fermentable carbon sources such as ethanol and glycerol. Aeration of cultures was done by agitation at 160 r.p.m. and was monitored spectrophotometrically at 600nm.

RNA isolation

Cells for RNA extraction were harvested at early- to mid-exponential growth phase, corresponding to OD_{600} between 0.25-0.40. One ml of the culture was collected and centrifuged at 1500x*g* at 4°C and cells were ressuspended in the same volume of deionised water at 4°C. After new centrifugation with the same conditions, supernatant was discarded and cells were frozen in liquid nitrogen and maintained at -80°C until use. RNA extraction was done using the hot acidic phenol method as described by Ausubel co-authors (1996). To inactivate RNases, all solutions used were prepared with DEPC-treated water [0.1% (v/v) and incubation for 16 h followed by autoclaving at 120ºC, 1 atm, 60 min] and reagents were nuclease-free. Glass labware was heat-treated at 200ºC for 16 h. Cells were ressuspended in 400µl of TES solution [10 mM tris·Cl, pH 7.5; 10 mM EDTA 0.5% (w/v) SDS] and 400µl of acid phenol (pH 5) was added followed by vigorous agitation in vortex for 10 sec. For complete disruption of cells and denaturation of RNases and other proteins, this mixture was incubated at 65ºC for 45 min with occasional vortexing. After heat incubation, the mixture was

placed in ice for 5 min and centrifuged in a microcentrifuge for 5 min at top speed, 4ºC. The upper aqueous phase was transferred to a new microcentrifuge tube and more 400µl of acid phenol were added. The mixture was vortexed and incubated, again, 5 min on ice and centrifuged in the same conditions. The upper aqueous phase was transferred to a new microcentrifuge tube and was extracted with 400µl of chloroform, followed by vigorous vortexing and centrifugation as described previously. The aqueous phase was transferred to a new microcentrifuge tube and precipitation of RNA was done by the addition of 40µl of 3M sodium acetate (pH 5.3) and 1ml of ice-cold 100% ethanol and incubated at -20ºC for, at least, 30 min. RNA was collected by centrifugation in a microcentrifuge 5 min at top speed, 4ºC, and washed with ice-cold 70% ethanol by brief vortexing and centrifugation. The RNA pellet was air-dried and dissolved in 50µl of nuclease-free water. RNA samples were kept at -20ºC for short-term storage and at -80ºC for long-term storage.

Because small amounts of DNA can be copurified with RNA that could act as target in subsequent PCR reactions, a DNase I incubation step was used. In DNase buffer (20 mM tris·HCl, pH 8.3; 50 mM potassium chloride; 20 mM magnesium chloride), 1 µg total RNA was incubated with 3 U DNase I, RNase-free in a total volume of 10 µl, at room temperature for 15 min. To prevent thermal degradation of RNA in the presence of magnesium ions (Barshevskaia *et al.*, 1987), 1 µl 25 mM EDTA was added and inactivation of DNase I was done by incubation at 65ºC for 10 min. RNA from this mixture is suitable for reverse transcription reaction and was kept at –20ºC.

Analysis of RNA

The concentration and purity of the RNA samples were determined by spectrophotometry. The optical density of convenient dilution of samples was measured at 260 nm and 280 nm. The A_{260}/A_{280} ratio was calculated for every sample and compared to reference values of 2.0±0.15 (Ausubel *et al.*, 1996; Farrell Jr, 1996) Samples which the A_{260}/A_{280} ratio lied between the higher and lower values were considered free of contaminants such as proteins, phenol and salts. Samples that did not match the reference values were purified by two extractions with acid phenol and chloroform and, afterwards, precipitated. The pellets were washed with ice-cold 70% ethanol, air-dried, dissolved in nuclease-free water and checked for absorbance at 260 nm and 280 nm. For samples that accomplished the purity criterion, the concentration was calculated with the formula: [RNA]µg/µl = A_{260} x dilution x 40.0 (Ausubel *et al.*, 1996; Farrell Jr, 1996).

Integrity of RNA was determined by agarose gel electrophoresis under denaturing conditions with formaldehyde. Gels were prepared with 1% (w/v) agarose, MOPS running buffer (40 mM MOPS, pH 7.0; 10 mM sodium acetate; 1 mM EDTA), 2.2 M formaldehyde, and 0.9 mg/ml ethidium bromide. Samples were heat denatured at 55°C for 15 min with MOPS running buffer, 2.2 M formaldehyde, and 50% (v/v) formamide. After heat denaturation, samples were immediately placed on ice and were added formaldehyde loading buffer 6x [1 mM EDTA; 0.25% (w/v) bromophenol blue; 50% (v/v) glycerol] to 1x final concentration. The amount of RNA loaded per lane was 0.5 to 10 µg and electrophoresis were run at 5 V/cm until bromophenol blue has migrated two-

thirds of the gel. Finally, gels were visualised and photographed under UV radiation.

Relative quantitative reverse transcriptase polymerase chain reaction (RT-PCR)

Expression studies on *GUP1* and *GUP2* were done by determination of the relative amounts of mRNAs in total RNA extracts by relative quantitative RT-PCR. First strand cDNA was synthesized using the Thermoscript™ RT-PCR System (Life Technologies™) from 100 ng of total RNA that has been confirmed to be pure and non-degraded. RNA was firstly denatured by incubation at 65ºC for 5 min with 50 ng random hexamers and placed on ice until use. The reaction mixture of 20 µl was transferred to cDNA synthesis buffer (50 mM tris acetate, pH 8.4; 75 mM potassium acetate; 8 mM magnesium acetate), 5 mM DTT, 40 U RNaseOUT™ (inhibitor of RNase activity of ThermoScript RT™), 1 mM dNTP mix, and 15 U of the reverse transcriptase ThermoScript RT™. Reverse transcription reaction was done in a pre-heated thermal cycler at 25ºC programmed to 25ºC, 10 min for random hexamers annealing, 50ºC, 50 min for reverse transcription, and 85ºC, 5 min for denaturation of ThermoScript RT™. Complementary RNA was degraded by incubation of cDNA with 2U RNase H, at 37ºC, 20 min in order to improve sensitivity. cDNA samples were stored at -20ºC.

Multiplex PCR reactions were performed in 2 µl of cDNA as template, with 50 mM $MgCl_2$, 10 mM dNTP mix, 10 µM of each primer, 4 µl 18S primer/Competimer™ mix (for *GUP1*: 2 µl 5 µM 18S rRNA primer pair/8 µl Competimers™; for *GUP2*: 1 µl 5 µM 18S rRNA primer pair/9 µl Competimers™), 2 U Platinum *Taq* DNA polymerase, 20 mM tris-HCl (pH 8.4), 50 mM KCl in a total volume of 50 µl. The sequence of primers are presented in table IV.I for *GUP1*, *GUP2* and internal standard 18S rRNA from commercial source (QuantumRNA™ 18S Internal Standards, Ambion), which included Competimers™ consisting in 3'-end-modified 18S rRNA primers that cannot be extended by DNA polymerase.

Table IV.I. Sequence of primers used in relative quantitative RT-PCR.

Primer	Sequence (5' to 3')
RTGUP1f (*GUP1* forward primer)	CGTGGTTCCCTTGATGTTCT
RTGUP1r (*GUP1* reverse primer)	TATGGGGTGTGCAGCAGTTA
RTGUP2f (*GUP2* forward primer)	TTCAAAAGGCGACAAAGGAT
RTGUP2r (*GUP2* reverse primer)	TCGTTCCTTTTCCTCCAGAA
18S rRNA internal standard forward primer	AGGAATTGACGGAAGGGCAC
18S rRNA internal standard reverse primer	GCACATCTAAGGGCATCACA

PCR program for *GUP1* was as follows: 94ºC, 30 sec; 55ºC, 30 sec; 72ºC, 1 min in a total of 29 cycles, being the first denaturation step of 2 min and the last extension step of 5 min. For *GUP2*, the program was similar except for annealing temperature of 57ºC and the number of cycles of 34. PCR products were separated by 1.5% agarose gel electrophoresis (5 V/cm), stained with ethidium

bromide and photographed under UV radiation. Photos were digitalized and densitometric analysis was performed using NIH Image 1.60 software. For each sample, the ratio between the fluorescence of the target gene and the internal standard was used to overcome variability between samples such as RNA quality, RNA quantification errors, and random tube-to-tube variation in PCR and reverse transcription reactions. Results are mean values of, at least, three independent experiments. Photos from representative experiments are shown.

Detection of contaminating DNA in all RNA samples was performed including a sample in which ThermoScript™ RT was omitted in RT-PCR. The absence of amplification, under these conditions, meant absence of genomic DNA amount sufficient for successful amplification by PCR.

Quantification of intracellular solutes by high performance liquid chromatography (HPLC)

Cells from 100 ml aliquots of cultures were harvested by centrifugation (7400x*g*, 5 min, 4ºC) and washed twice by resuspension in ice-cold ultra-pure water and centrifugations under the same conditions. The pelleted cells were stored at -80ºC or immediately resuspended in 2 ml ice-cold ultra-pure water. Cells were disrupted and proteins were precipitated with 2 ml 8% (w/v) perchloric acid with homogenisation and incubation 45 min at 70ºC with agitation in vortex every 10 min. Cell debris were separated by centrifugation at 10,000x*g*, 4ºC for 30 min and supernatant was collected and stored at -20ºC. Dry weight was determined by filtration of 20 ml of the culture through 0.2 µm pore membranes (Schleicher & Schuell). The membrane was washed with 20 ml ice-cold deionised water and was dried at 80ºC for 24 hours. For injection on HPLC column, samples were diluted with equal amount of internal standard (10 g/l arabinose). Results are mean values of, at least, three independent experiments.

The retention time of glycerol 3-phosphate was analysed in order to prevent misidentification of the glycerol peak. To evaluate the leakage of intracellular solutes during the wash steps of cells, an aliquot of supernatant of the first wash step of every sample was stored, treated the same way as the other samples, and analysed.

Results

General considerations on RT-PCR

Several evidence indicated beforehand that the expression of *GUP1* and *GUP2* was probably very low. In fact, for glycerol uptake, V_{max} determined was very low when compared to other active uptake systems (see Chapter III). In addition, phenotypes revealed in agar plates to test utilisation of glycerol as carbon and energy source and glycerol-mediated recovery from salt stress, were not very pronounced (Holst *et al.,* 2000). Most importantly, assays with *GUP1* and *GUP2* promoter fusions with *lacZ* have shown that their activity is very weak

being, the one of *GUP2* around 10-fold weaker than the one of *GUP1* promoter (Holst *et al.,* 2000). These expression results are in accordance with the extremely low codon bias index calculated for *GUP1* and *GUP2* (see Chapter III). Therefore, for analysis of expression of these genes, a sensitive method should be selected. Reverse transcription coupled with polymerase chain reaction (RT-PCR) is a method developed after hybridisation-based techniques have been applied to expression analysis (Chelly *et al*., 1988; Becker-André and Hahlbrock, 1989; Wang *et al.,* 1989) and one of its remarking features is the high sensitivity, which is provided by the amplification step. This characteristic has proven to be extremely valuable for analysis of clinical samples because of the usual limited amount of biological material, as is the case of biopsies (Noppen *et al.,* 1996; Gaynor *et al.,* 1996). Although Northern analysis remains the widest method to study gene expression in yeasts, because the amount of biological material is not usually critical, RT-PCR with modifications has been increasingly used. For highest sensitivity, detection of amplicons can be made, after size separation by electrophoresis, by Southern analysis (Li *et al.,* 1998b) or by autoradiography when nucleotides or primers used for PCR are radiolabelled (Naglik *et al.,* 1999; Reeder *et al.,* 1999). Naglik and co-workers (1999) reported the detection of *SAP2* mRNA in *Candida albicans* from as little as 1 pg total RNA.

The RT-PCR technique allows several modifications that can be followed to estimate absolute or relative quantities of mRNA's and in order to adjust sensitivity, accuracy, and precision. The most common one is competitive RT-PCR in which a standard, in different dilutions, competes with a constant amount of target cDNA for reagents in independent amplification reactions, allowing absolute quantification of target mRNA when 1:1 molar ratio of standard/target mRNA is obtained after separation by electrophoresis. On the other hand, non-competitive kinetic RT-PCR can be employed. In relative quantitative RT-PCR (also called semi-quantitative RT-PCR) an internal standard, usually cDNA from mRNA of a housekeeping gene, is co-amplified with the target cDNA in a non-competitive fashion and relative amounts of the target mRNA are obtained by comparing with the internal standard amplicon (Harting and Wiesner, 1997; Saric and Shain, 1997; Chen *et al*., 1999; Masuda *et al.,* 2000). Kinetic RT-PCR can be done with or without the use of any standard with absolute quantification of initial template concentration simply by kinetic analysis of PCR. These kinetic approaches were greatly improved with systems that allow detection of amplicon formation during PCR (real-time kinetic RT-PCR), avoiding the error generating steps of processing for end-point detection or removal of aliquots.

Relative quantitative RT-PCR was used to study the expression of *GUP1* and *GUP2*. For first strand cDNA synthesis, a modified avian reverse transcriptase lacking RNase H activity and with high thermal stability (ThermoScript RT™, GibcoBRL) was used. The reaction temperature of 50ºC was chosen to avoid secondary structures of mRNAs that could prevent primer annealing and/or fixation of the reverse transcriptase to the template (Myers and Gelfand, 1991; Mallet *et al.,* 1995; Shimomaye and Salvato, 1989; Wang *et al.,* 1992; Brooks *et al.,* 1995; Freeman *et al.,* 1996; Reeder *et al.,* 1999). Primers for reverse transcriptase reaction were non-specific (random hexamers) to obtain cDNA from total RNA, allowing multiplex PCR on cDNA synthesised.

Internal standard chosen to be co-amplified with target cDNA was 18S rRNA, for which weak variations among eukaryotic cells have been reported (Thellin *et al*., 1999; Goidin *et al.,* 2001; Zhou *et al.,* 2002). Large amounts of 18S rRNA are

present in cells, which should cause rapid depletion of reagents in the amplification reactions, leading to an incomparable higher product formation than, presumably, the target cDNA. In this case, the levels of amplicons should not ever be comparable, hampering the relative quantification of the amplicon under study. To circumvent this situation, competimer technology was used, allowing modulation of internal standard amplification efficiency to levels comparable to those of target cDNA. The competimers used were 18S rRNA primers for PCR with the same sequence as those used for internal standard but with a modification at their 3' ends to prevent extension by the polymerase. This way, these competimers will decrease the amount of amplicon by competition with18S rRNA primers for the same template, which can be adjusted by manipulating the ratio of primer/competimer to use in PCR.

To ensure high specificity and sensitivity in PCR, a "hot-start" method was utilised. A recombinant *Taq* DNA polymerase (Platinum™ *Taq* DNA polymerase) complexed with a specific antibody inhibiting its polymerase activity was used. Therefore, extension of primers that could anneal non-specifically would not occur during assembly of PCR reactions at room temperature until the denaturing step of the first PCR cycle. At 94ºC the antibody denatures, releasing the fully active *Taq* DNA polymerase that can only extend primers annealed at the programmed temperature.

The kinetics of the reverse transcription reaction is stated in Equation 1 (Freemann *et al.,* 1999):

$$[cDNA]=[RNA]xEfficiency \qquad [Eq.\ 1].$$

For maximal efficiency and full conversion of RNA to cDNA, reverse transcription conditions were followed strictly according to manufacturer's recommendations and RNA's were quantified carefully and controlled for integrity by visualisation of ethidium bromide stained gels after separation by electrophoresis under denaturing conditions (see Materials and Methods). For quantitative results to be proportional to the amount of initial template concentrations, analysis of PCR products should be done in exponential phase of amplification both for target and internal standard. Only during this phase of amplification reaction, amplicon formation increases exponentially and is dependent of initial number of target molecules and amplification efficiency, which is constant and maximal. In this phase, product formation (P), after a given number of cycles (n), is directly proportional to the initial amount of target (T) and amplification efficiency (E) according to Equation 2:

$$P=T(1+E)^{n}, \qquad [Eq.\ 2]$$

(Freemann *et al.,* 1999; Siebert, 1999). While reagents keep in excess, Equation 2 is applicable to predict the course of the reaction until either (i) one of the reagents becomes limiting and/or amplification products accumulate and compete with primers for annealing, (ii) the amount of active polymerase becomes limiting, (iii) or the formation and accumulation of polymerase inhibitors impedes its activity. When at least one of these factors is present, amplification efficiency slows down, and a plateau of formation of amplicon is reached (Siebert, 1999). Therefore, comparison between samples for the same amplicons is only feasible during the linear range of amplification, because amplification efficiencies should remain constant. However, due to the exponential nature of

PCR, random errors are amplified as well, leading to rather high tube-to-tube variation and amplification efficiencies. The co-amplification of an endogenous internal standard, which is known to be expressed at constant levels among samples to be compared, allows compensation for this variation and, in addition, for variations in the reverse transcription reaction caused by impurities affecting efficiency and erroneous RNA quantification (Freemann *et al.,* 1999).

Optimisation of RT-PCR

Linear range of amplification was determined for each target (*GUP1* and *GUP2*) and internal standard (18S rRNA). Reverse transcription was made in a RNA sample from strain W303-1A grown up to mid-exponential growth phase using ethanol as carbon and energy source, ensuring derepression of glycerol active uptake (Lages and Lucas, 1997). PCR reactions were always setup with cocktail mixtures including all common components in order to minimise pipeting errors. *GUP1* target was co-amplified with a segment of 18S rRNA in a multiplex PCR, and 10 µl aliquots of reaction mixes were removed at different convenient cycle numbers and analysed. Plots of PCR product fluorescence versus cycle number revealed linearity of amplification up to cycle number 30 for both *GUP1* and 18S rRNA (Fig. IV.1/A and B). The same analysis applied for *GUP2* and 18S rRNA, revealed linearity up to cycle number 36 (Fig. IV.1/C and D). In all cases, the correlation calculated was 0.99. These results allowed to select the cycle number at which reactions should stop for analysis of PCR products in order to obtain data proportional to the initial levels of any molecule to be amplified: target and internal standard. Hence, for *GUP1*, the end for PCR was chosen to be at cycle 29 while for *GUP2* was cycle 34. Importantly, the ratios between fluorescences of target and internal standard through the linear range of both reactions were rather invariant, with mean values of 0.97±0.12 for *GUP1*/18S rRNA and 5.03±0.42 for *GUP2*/18S rRNA. This means that amplification efficiencies are identical as regression lines run in parallel and target/standard ratios will not differ through the linear range of reactions, allowing meaningful comparisons between target and standard.

To determine the limits of detection of variations of mRNA and the upper and lower amounts of total RNA allowing quantification in RT-PCR experiments, known as “dynamic range”, different amounts of input total RNA were assayed. The same sample used for linear range determinations was used for dynamic range studies, with RT-PCR reactions performed according to previous optimisations and the number of cycles in multiplex PCR within the linear phases of amplification (29 cycles for *GUP1*/18S rRNA and 34 cycles for *GUP2*/18S rRNA). Linear correlations between input RNA (from 1µl to 4 µl, corresponding to 5 ng to 20 ng input RNA) and amplicons were found (Fig. IV.2), meaning that, at least, half and double mRNA molecules in relation to levels of derepressed cells can be detected. Nevertheless, higher levels could be detected as can be seen by the fluorescence intensity of bands corresponding to the highest amount of input RNA’s (Fig. IV.2/A and C). The determinations of linear and dynamic ranges for *GUP1*/18S rRNA and *GUP2*/18S rRNA were possible with detection of amplification products based on ethidium bromide staining. As stated above, for maximal benefit of RT-PCR sensitivity, the detection of PCR products is important and can be done by autoradiography when nucleotides or primers are radiolabelled. Silver staining and SYBR® Green (Molecular Probes) allow much

more sensitivity than ethidium bromide as well, but for the purposes of this study ethidium bromide staining showed sufficient sensitivity.

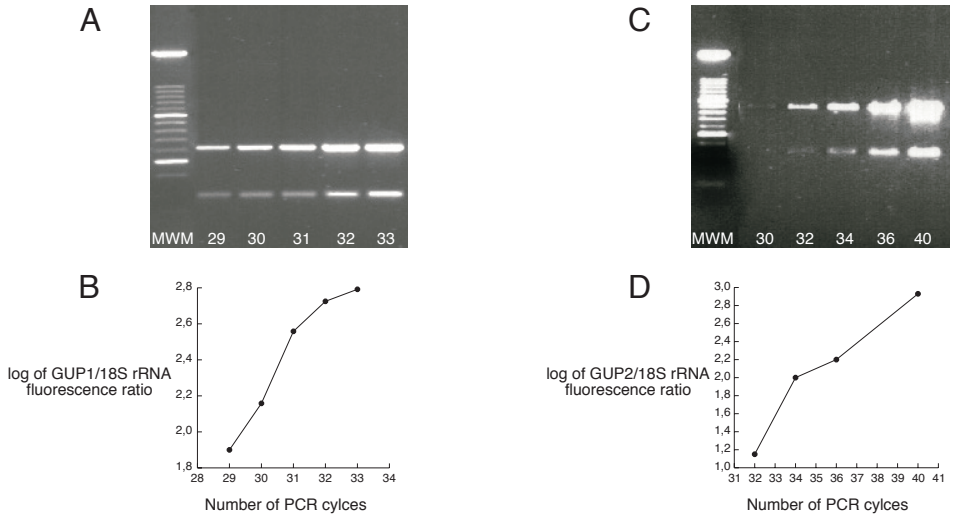

Figure IV.1. Cycle number dependence of amplification of *GUP1* (A and B) and *GUP2* (C and D) in RT-PCR. RT-PCR prodcuts for each cycle number were separated by agarose gel electrophoresis, stained with ethidium bromide and photographed (A and C). A: upper bands correspond to *GUP1* and lower bands correspond to 18S rRNA. C: upper bands correspond to *GUP2* and lower bands correspond to 18S rRNA (MWM: molecular weight marker). Quantification is given by the target gene/18S rRNA fluorescences ratio and plotted against cycle number (B and D).

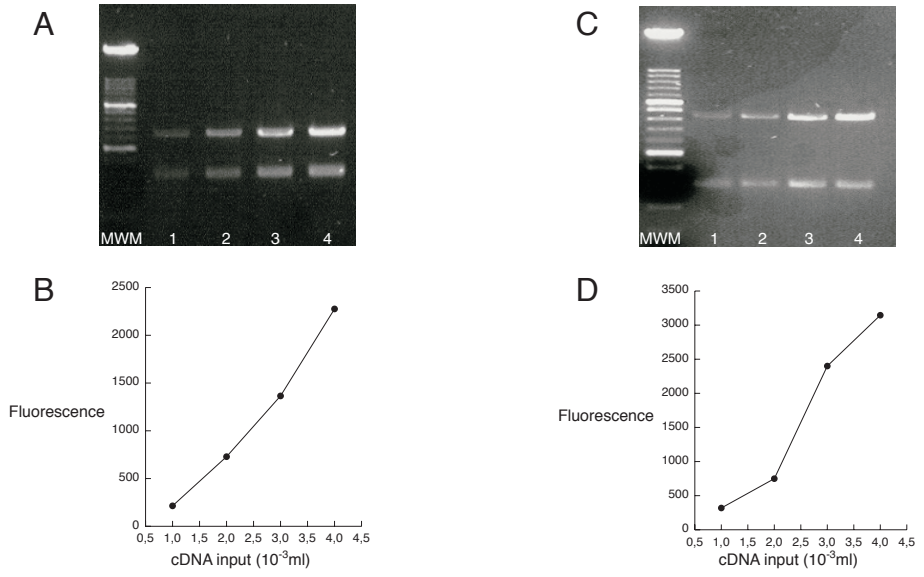

Figure IV.2. cDNA input dependence of amplification of *GUP1* (A and B) and *GUP2* (C and D) in RT-PCR. RT-PCR prodcuts for each assay performed with 1 μl, 2 μl, 3 μl and 4 μl input cDNA were separated by agarose gel electrophoresis, stained with ethidium bromide and photographed (A and C). A: upper bands correspond to *GUP1* and lower bands correspond to 18S rRNA. C: upper bands correspond to *GUP2* and lower bands correspond to 18S rRNA (MWM: molecular weight marker). B and D: fluorescence intensity of amplification of *GUP1* (B) and *GUP2* (D) plotted against cDNA input in RT-PCR reactions.

Relative quantification of GUP1mRNA and GUP2mRNA by relative quantitative RT-PCR

As mentioned in Introduction, glycerol active uptake, attributed to *GUP1*, is detected exclusively in ethanol-grown cells (Lages and Lucas, 1997), although phenotypes of salt recovery, in the presence of glucose, and glycerol assimilation deficiency have been detected in the *gup1* mutant strain (Holst *et al.,* 2000). For *GUP2*, the only situation in which glycerol uptake could be assigned to was under salt stress and using a strain unable to synthesise glycerol (*gpd1gpd2* genetic background) (Holst *et al.,* 2000). So, the first approach in expression studies was to assess whether, exists any correlation between physiological data and steady-state mRNA levels in any of these physiological conditions. mRNA levels were measured at early- to mid-exponential cells growing on glucose and on ethanol by relative quantitative RT-PCR (Fig. IV.3). Additionally, samples were harvested from batch cultures with glucose when cells were at the diauxic shift from fermentative to respiratory metabolism and at post-diauxic respiratory metabolism (Fig. IV.3). Results of expression of *GUP1* and *GUP2* in exponentially growing cells on glucose and on ethanol do not match with experimental determinations of glycerol uptake. Surprisingly, glucose-grown cells presented mRNA levels comparable to those of ethanol-grown cells, pointing to regulation of glycerol uptake at translational or post-translational levels. The expression determined along growth on glucose up to respiratory metabolism decreases slightly for both genes, although with levels that do not differ significantly. A striking exception is the low level of *GUP1* expression in the diauxic shift that might be connected to reprogramming of cell metabolism.

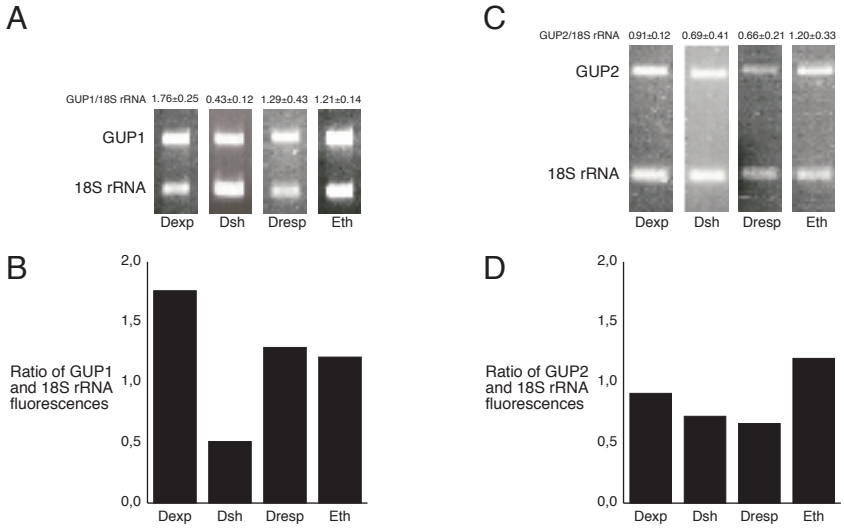

Figure IV.3. Relative quantitative RT-PCR of transcripts of *GUP1* (A and B) and *GUP2* (C and D) in cells of *S. cerevisiae* strain W303-1A grown in YPD and harvested at mid-exponential growth phase (Dexp), diauxic shift (Dsh), post-diauxic shift (Dresp) and grown in YPE and harvested in mid-exponential growth phase (Eth). RT-PCR prodcuts of each assay were separated by agarose gel electrophoresis, stained with ethidium bromide and photographed (A and C). Quantification is given by the ratio of target gene/18S rRNA fluorescences and plotted against growth conditions (B and D).

The influence of salt stress on *GUP1* and *GUP2* expression was investigated as well. Physiological data available indicates that glycerol active uptake is not induced by salt stress (Lages and Lucas, 1997), although *gup1* mutant strain presents slower growth in media with 1 M NaCl and 10 mM glycerol than the wild-type (Holst *et al.,* 2000). As for *GUP2*, no phenotype had been assigned to this gene when testing the correspondent mutants in wild-type genetic background (Holst *et al.,* 2000). Cells collected in early- to mid-exponential growth phase were analysed for *GUP1* and *GUP2* mRNA's relative content by RT-PCR (Fig. IV.4). In rich media with glucose supplemented with 1 M NaCl alone or with further supplementation with 15 mM glycerol, mRNA levels were constant for both *GUP1* and *GUP2*. Therefore, no induction of expression for both genes was observed by extracellular glycerol under salt stress. In addition, no significant differences were observed from salt stress media, with or without 15 mM glycerol, to glucose exponential phase cells (Figs. IV.3 and IV.4). Here, a match with glycerol uptake data was found in terms of equal behaviour between cells grown in glucose with or without salt stress (regardless the presence of 15 mM glycerol in salt media). The transcriptional activity is the same in all cases with simultaneous lack of detection of glycerol active uptake.

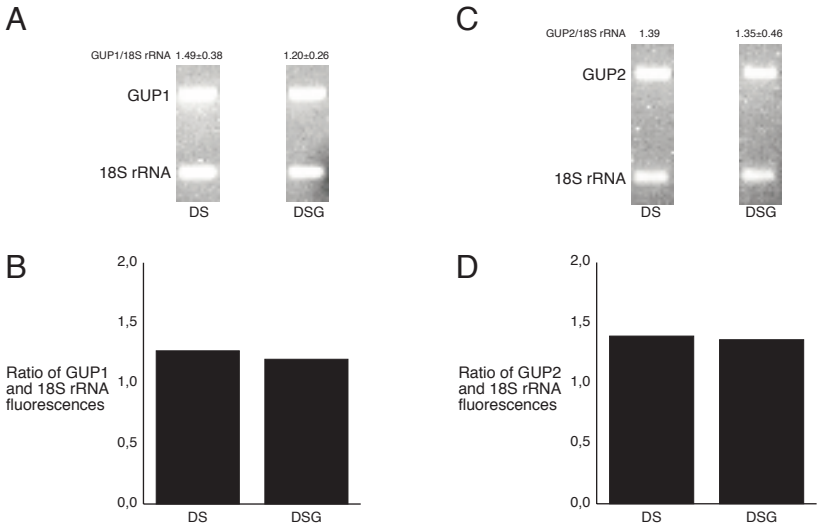

Figure IV.4. Relative quantitative RT-PCR of transcripts of *GUP1* (A and B) and *GUP2* (C and D) in cells of *S. cerevisiae* strain W303-1A grown in YPD supplemented with 1M NaCl (DS) or further supplemented with 15 mM glycerol (DSG) and harvested at mid-exponential growth phase. RT-PCR prodcuts of each assay were separated by agarose gel electrophoresis, stained with ethidium bromide and photographed (A and C). Quantification is given by the ratio of target gene/18S rRNA fluorescences and plotted against growth conditions (B and D).

A striking feature of glycerol transport is the high uptake in a *gpd1gpd2* strain grown in glucose with 1 M NaCl and 15 mM glycerol, which was attributed to Gup2p (see Chapter III). An interaction of *GPD1* and/or *GPD2* with *GUP2*, leading to increased expression, could explain this result. To assess this possibility, transcript analysis by RT-PCR was applied to *gpd1gpd2* strain under conditions of salt stress in glucose-based media as done before for the wild-type (Fig. IV.5). For *GUP1*, expression did not vary significantly with salt stress both in W303-1A (Fig. IV.4) or gpd1gpd2 (Fig. IV.5) strains and between salt stress media with or without 15 mM glycerol for *gpd1gpd2* strain (Fig. IV.5). On the other hand, expression of *GUP2* is higher in salt stress media for *gpd1gpd2* (Fig.

IV.5) strain than for W303-1A (Fig. IV.4). Therefore, induction of expression of *GUP2* is apparently triggered by salt stress with impairment in glycerol synthesis. For glucose-grown cells, *GUP2* transcript levels are higher as well in *gpd1gpd2* than in W303-1A (Figs. IV.3 and IV.5), while transcript levels of *GUP1* are not significantly changed for *gpd1gpd2* cells, although with a slight decrease (Figs. IV.3 and IV.5). Therefore, impairment in glycerol synthesis alone seems to increase transcript levels of *GUP2*. With only one exception, no correlation was found between transcript levels and glycerol uptake experimental detection. The exception is *GUP2* of strain *gpd1gpd2* in salt stress medium with glycerol for which the highest V_{max} was measured (see Chapter III), suggesting that a critical value was reached for the appearance of *GUP2*-dependent glycerol uptake.

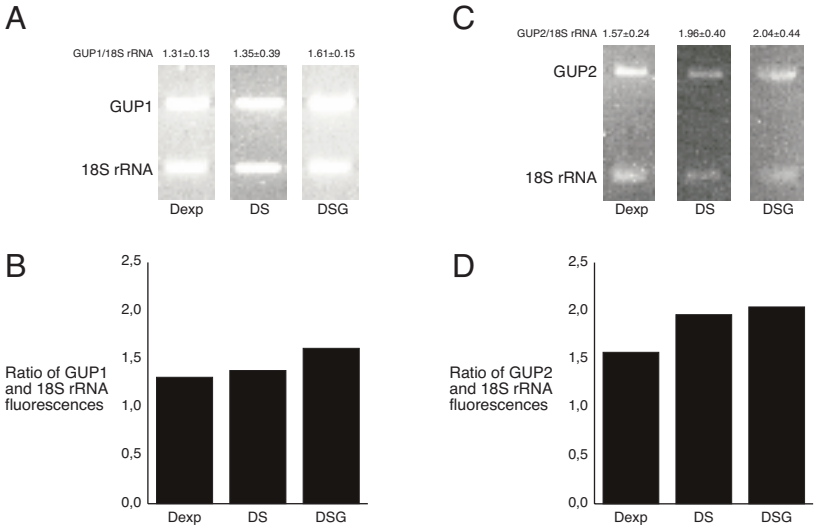

Figure IV.5. Relative quantitative RT-PCR of transcripts of *GUP1* (A and B) and *GUP2* (C and D) in cells of *S. cerevisiae* strain YSH642 (*gpd1gpd2*) grown in YPD (Dexp) or YPD supplemented with 1M NaCl (DS) or with further supplementation with 15 mM glycerol (DSG) and harvested at mid-exponential growth phase. RT-PCR prodcuts of each assay were separated by agarose gel electrophoresis, stained with ethidium bromide and photographed (A and C). Quantification is given by the ratio of target gene/18S rRNA fluorescences and plotted against growth conditions (B and D).

Quantification of intracellular glycerol and trehalose by HPLC

The unexpected levels of transcription of *GUP1* and *GUP2* with simultaneous lack of detection of glycerol uptake in glucose-grown cells can be explained by a possible feedback inhibition of the transporter by its own substrate: glycerol. When *S. cerevisiae* cells are fermenting, redox balance is maintained by synthesis of glycerol, oxidating the excess of NADH resulting from biomass production (van Dijken and Scheffers, 1986). If intracellular levels of glycerol reach a critical value, an inhibition of the transporter exerted simply by direct interaction on the transporter protein could occur. This kind of regulation was proposed by Séron and co-workers (1999) for uracil and uracil permease and by Robinson and co-workers (1996) for inositol and its permease. Therefore, whenever high intracellular levels of glycerol are reached, glycerol uptake would be inhibited, despite a constitutive transcription of *GUP1* and *GUP2*. The intracellular levels of glycerol were measured by HPLC in the same strains and

growth conditions tested for RT-PCR in order to investigate this type of regulation in *GUP1* and *GUP2*.

Intracellular levels of glycerol in W303-1A strain reached 508 mM under salt stress (without added glycerol), which is an enormous increase when compared with glucose-grown cells without salt stress (Fig. IV.6). The fact that intracellular glycerol is undetectable in glucose-grown cells is presumably due to excretion because, under fermentative metabolism, yeast cells synthesise glycerol for redox balance (van Dijken and Scheffers, 1986) and in the absence of salt stress the Fps1p channel is opened (Tamás *et al.,* 1999). When cells are exposed to salt stress with 15 mM glycerol in the medium, intracellular glycerol is about half the value determined for salt stress without glycerol supplementation. In *gpd1gpd2* strain, as expected, intracellular glycerol is significantly lower than the wild-type. Unexpectedly, this strain exhibits measurable intracellular glycerol under salt stress, suggesting some capability of glycerol synthesis in this strain. Actually, this strain is capable of growing under severe salt stress (1 M NaCl) with and without supplementation with 15 mM glycerol (Fig. IV.7). While in the first case, glycerol can be taken up by Gup2p and Gup1p for intracellular accumulation (see Chapter III), without external glycerol supplementation only synthesis can contribute for its accumulation. This can explain the marked difference in lag phase for both conditions (Fig. IV.7), being needed for *gpd1gpd2* a longer adaptation period for glycerol synthesis when it is absent in the medium.

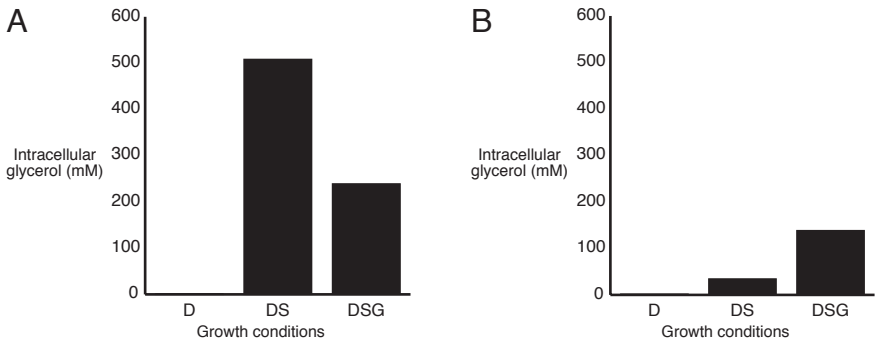

Figure IV.6. Levels of intracellular glycerol determined by HPLC in *S. cerevisiae* strains W303-1A (A) and YSH642 (*gpd1gpd2*) (B) cells grown in YPD (D), YPD supplemented with 1 M NaCl (DS) and with further supplementation with 15 mM glycerol (DSG).

The possible feedback inhibition by glycerol on its own transporter could explain the lack of uptake activity detected in W303-1A when grown in glucose-based medium with salt stress with or without 15 mM glycerol. The very high intracellular glycerol levels observed in these two cases (Fig. IV.6) are consistent with this assumption. However, in *gpd1gpd2* mutant strain grown under salt stress with supplementation of glycerol, substantial amounts of intracellular glycerol were observed as well and in opposition to the expected since glycerol transport not only was detectable but its V_{max} was the highest measured ever. Actually, the high intracellular amounts of glycerol could be the consequence of the high transport activity observed. Therefore, Gup1p and Gup2p seem to lack this regulation of feedback inhibition by the substrate.

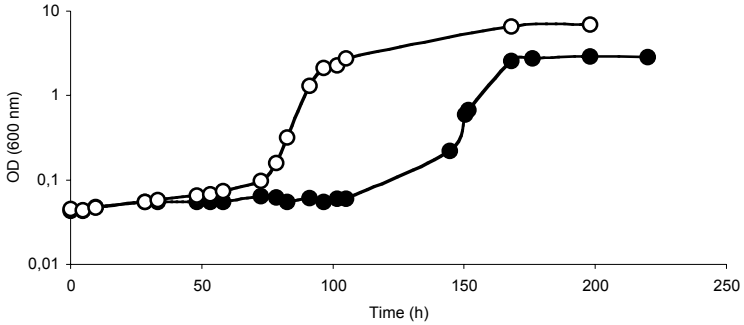

Figure IV.7. Growth curves measured as optical density at 600 nm of *S. cerevisiae* strain YSH642 (*gpd1gpd2*) in YPD medium with 1 M NaCl (●) and with further supplementation with 15 mM glycerol (○).

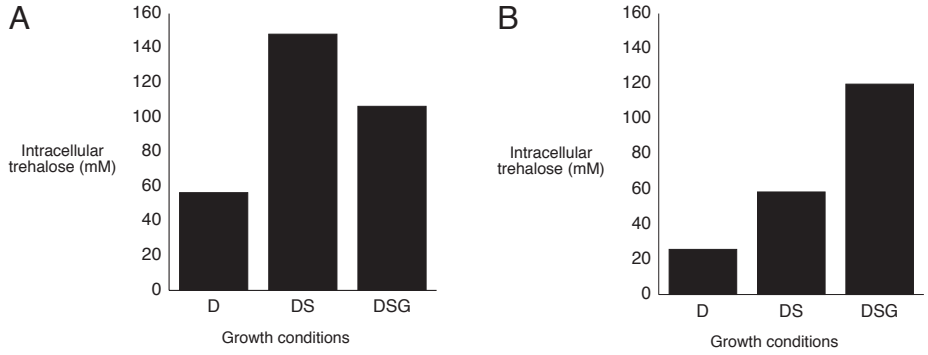

Figure IV.8. Levels of intracellular trehalose determined by HPLC in *S. cerevisiae* strains W303-1A (A) and YSH642 (*gpd1gpd2*) (B) cells grown in YPD (D), YPD supplemented with 1 M NaCl (DS) and with further supplementation with 15 mM glycerol (DSG).

It is interesting to note the ability of the *gpd1gpd2* mutant to grow under salt stress, together with the unexpected intracellular accumulation of glycerol. This intracellular accumulation of the compatible solute is not as high as the one observed in the same strain under salt stress with 15 mM glycerol. However, the growth rate is even higher in medium without supplementation of glycerol ($0.162h^{-1}$) than with supplementation ($0.112h^{-1}$) being, the lag phase much lower in this case (Fig. IV.7). Therefore, the glycerol content alone, apparently, does not explain the adaptation of this mutant strain to salt stress. Another metabolite known to accumulate during adaptation to salt stress is trehalose (MacKenzie *et al.,* 1988). The action of trehalose has been reported to be as a non-osmoregulatory protector under general stress conditions (Mager and Varela, 1993) and to estimate whether the trehalose contribution to salt stress tolerance in *gpd1gpd2* mutant is higher than in W303-1A, the intracellular content was determined by HPLC (Fig. IV.8). Consistently with stress protectant functions, for each strain, the intracellular content is higher in salt stressed cells than in glucose-grown cells. However, trehalose concentrations attained in *gpd1gpd2* are not as high, comparing with W303-1A strain, as it would be expected if trehalose would replace glycerol as an osmotic protectant.

Discussion

Expression analysis of *GUP1* and *GUP2* revealed constant presence of transcripts for both genes under conditions tested, indicating constitutive transcription activity for cells growing on, at least, glucose, ethanol, and in glucose under salt stress. Interestingly, for *GUP1* and *GUP2*, no induction of expression is observed in batch cultures with glucose when glucose is depleted (diauxic shift), or when cells are respiring ethanol produced earlier by glucose fermentation (Fig. IV.3). This is in agreement with mRNA levels at exponential growth phase in ethanol-grown cells (for *GUP1*) compared with those of glucose-grown cells. The striking low expression level of *GUP1* in diauxic shift cells, compared to fermentative and respiratory cells in batch cultures with glucose as carbon and energy source, could mean that *GUP1* is necessary for both types of energetic metabolism and, moreover, a possible additional function for Gup1p due to the fact that no glycerol uptake is detected in cells grown on glucose. A slight progressive decrease of expression in cells shifting from fermentative metabolism to the diauxic shift, in which occurs an arrest of fermentation and reprogramming of gene expression for respiratory metabolism was found, as well, by DeRisi and co-workers (1997) by microarray analysis. Transcript levels of *GUP2*, under the same conditions, analysed by microarrays match with RT-PCR analysis in this work (Fig. IV.3), pointing to progressive decrease when cells enter the diauxic shift. For *GUP1*, lower levels of transcripts in the diauxic shift and post-diauxic growth were observed by DeRisi and co-workers (1997) and Gasch and co-workers (2000), which is corroborated with results in this work. However, these authors reported an initial induction, which corresponds actually to exponential growth phase in relation to early-exponential phase that has not been determined by RT-PCR in this work. The decrease in steady-state levels of transcripts of *GUP1* and *GUP2* when cells of a batch culture on glucose enter diauxic shift and enter respiratory metabolism, suggest an involvement in fermentative metabolism than in respiratory metabolism. Nevertheless, the decrease in transcript levels of *GUP1* during diauxic shift and further increase for respiratory metabolism, indicate an involvement in this metabolism that cannot be disregarded.

The influence of a non-fermentable carbon source on steady-state levels of transcripts of *GUP1* and *GUP2* was determined by microarrays as well in an independent work (Gasch *et al.,* 2000). With the exception of yeast strain, the experiments were quite similar. Nevertheless, results for *GUP1* transcription by microarrays, with a slight increase of mRNA for ethanol-based medium, did not match exactly with RT-PCR results in this work (Fig. IV.3). For *GUP2*, in two microarray experiments for the same growth conditions, a slight repression and constant transcription activity were reported by Gasch and co-workers (2000), while in this work no significant changes were observed. The statistical significance of data generated by microarrays and RT-PCR is still a matter of debate and improvement (Freeman *et al.,* 1999; Siebert, 1999; Baldi and Long, 2001; Herwig *et al.,* 2001), which might explain non coincident results. However, it is clear from both experimental approaches that the *GUP1*-dependent glycerol uptake in ethanol-grown cells is not due to an increase in transcription activity since an induction is observed, comparing with control samples, for both glucose- and ethanol-grown cells in microarrays experiments (Gasch *et al.,* 2000) and mRNA levels are comparable between these samples in RT-PCR (Fig. IV.3).

The fact that *GUP1* is involved in glycerol-mediated recovery from salt stress, as demonstrated by Holst and co-workers (2000), together with the lack of glycerol uptake (Lages and Lucas, 1997) and the existence of *GUP1* mRNA's under the same physiological conditions (Fig. IV.4), suggests an additional role for Gup1p. In this case, contrarily to the case of diauxic shift (Fig. IV.3), a role specifically connected with salt stress response is strongly suggested. Therefore, two additional roles for *GUP1*, besides glycerol active uptake, are suggested by data from glycerol uptake assays, growth in agar plates, and RT-PCR: involvement in fermentative and respiratory metabolisms and salt stress response. One possibility could be a regulatory function, as suggested before by *in silico* analysis (see Chapter III) over genes or proteins that could act directly in these energetic metabolisms and in salt stress response. This means that *GUP1* could regulate glycerol uptake rather than actually transport this molecule. On the other hand, a sensorial role can explain these results as well and, mainly, the constitutive expression causing, presumably, the permanent presence of Gup1p in the plasma membrane for sensing osmotic stress or glycerol need for metabolism in order to trigger glycerol uptake by the actual transporter. The same could be applied to *GUP2*, taking in consideration the constant presence of transcripts in the conditions tested. Nevertheless, the striking results of higher transcript levels in salt stress medium with glycerol in *gpd1gpd2* strain compared with other conditions for the same strain and with W303-1A for the same medium and for glucose medium, suggest that, at least *GUP2* is involved in salt stress response and fermentation of glucose. Moreover, the match of glycerol uptake detection in glucose-based medium and higher levels of *GUP2* mRNA's supports the transporter role while the lack of glycerol uptake in glucose medium, even with higher transcript levels, seems to support the sensor or regulatory roles. Genome-wide expression analyses by microarrays were used to study expression reprogramming of yeast cells when facing an osmotic shock (Rep *et al*., 2000; Posas *et al.,* 2000; Gasch *et al.,* 2000; Causton *et al*., 2001; Yale and Bohnert, 2001). Observations of transcription induction of *GUP1* by osmotic shock of 1 M NaCl (Causton *et al.,* 2001; Yale and Bohnert, 2001) and absence of induction or repression by 0.4 M, 0.5 M, 0.7 M, and 0.8 M NaCl (Rep *et al.,* 2000; Posas *et al.,* 2000) were reported. Osmotic shock by sorbitol was found to not change expression of *GUP1* as well at 0.95 M (Rep *et al.,* 2000), 1 M (Gasch *et al.,* 2000), and 1.5 M (Causton *et al.,* 2001). For *GUP2*, in any case an absence of induced or repressed expression by osmotic shock, by NaCl or sorbitol, was found. The threshold for induction of transcription seems to be 1 M NaCl for *GUP1*, which is not in agreement with RT-PCR results in this work. However, the experimental design for RT-PCR experiments was different since steady-state transcript levels of adapted cells to salt stress were determined. This distinction of transcriptional activity of *GUP1* as function of osmotic shock or long-term osmotic stress might constitute a major difference taking in consideration these results. In fact, dramatic changes in transcript profile follow shock stress, but with adaptation to new environmental conditions imposed by the stress, gene expression adjusts and a new steady-state is reached that can be only slightly different from the initial non-stressful conditions (Gasch *et al.,* 2000). Hence, according to a model proposed by Gasch and co-workers (2000), the induction of transcriptional activity of *GUP1* could provide rapid and probably small increase in Gup1p levels until adaptation to the osmotic stress. Subsequently, maintenance of Gup1p levels could be achieved by very small changes in transcript levels. The possibility of regulation at translational and post-translational levels should not be overlooked as such regulatory

mechanisms fit this model by providing the steady-state maintenance of Gup1p levels at the new conditions.

Ansell and co-workers (1997) reported that in *gpd1gpd2* strain, no detectable intracellular glycerol was found. Contrarily, in this work, a significant amount of glycerol was determined in this strain (Fig. IV.6/B). However, different conditions were used for these experiments: while Ansell and co-workers (1997) measured intracellular glycerol after 4 h adaptation to salt stress, here, measurements were done in exponentially growing cells under salt stress. This difference is very important since lag phase for this strain in glucose medium with 1 M NaCl lasts more than 100 hours (Fig. IV.7), therefore the adaptation of 4 hours is far from being complete for beginning growth. As stated in Results, this intracellular glycerol is apparently due to synthesis. Two reasons can be appointed to support this conclusion: the absence of detectable external glycerol, at least by HPLC (data not shown), and the long-term adaptation to begin growth. The lack of glycerol 3-phosphate dehydrogenase (EC 1.1.1.8) encoded by *GPD1*, which is the key enzyme for glycerol synthesis in response to osmotic stress (Larsson *et al.,* 1993; Albertyn *et al.,* 1994b; Ansell *et al.,* 1997), together with *GPD2* deletion, causes almost complete blocking of glycerol synthesis. However, an alternative pathway of glycerol metabolism might exist in *S. cerevisiae* that could be active to synthesise glycerol (Norbeck and Blomberg, 1997). This pathway would convert sequentially dihydroxyacetone phosphate to dihydroxyacetone and to glycerol. It was demonstrated to be active in *Schizosaccharomyces pombe* (May and Sloan, 1981; May *et al.,* 1982) and in *Zygosaccharomyces rouxii* (van Zyl *et al.,* 1991). Dihydroxyacetone kinase (EC 2.7.1.29) activity was detected successfully in *S. cerevisiae* and is encoded by *DAK1* and *DAK2* (Norbeck and Blomberg, 1997; Molin *et al*., 2003). However, phosphatase activity with dihydroxyacetone phosphate as substrate has not been demonstrated yet. The subsequent step is catalysed by glycerol dehydrogenase (aldo-keto reductase; EC 1.1.1.156) and is encoded by *GCY1* (Hur and Wilson, 2000; Hur and Wilson, 2001). So, this alternative pathway for glycerol synthesis is still not confirmed to exist in *S. cerevisiae*.

Transport regulation by direct binding of the intracellular substrate over its transmembrane transporter has been proposed for uracil and inositol (Robinson *et al.,* 1996; Séron *et al.,* 1999). Interestingly, both substances are excreted to the extracellular medium, indicating a tight control of intracellular levels (Séron *et al.,* 1999). Glycerol is excreted as well and intracellular levels are regulated, involving changes in transcription rates of several genes and, at least, the activity of a constitutively expressed gene, *FPS1* (Tamás *et al.,* 1999) encoding a glycerol channel (Luyten *et al.,* 1995). Therefore, such control proposed for uracil and inositol, by analogy, would seem likely to occur for glycerol transporters. Nevertheless, this is not the case because intracellular levels of glycerol in glucose-grown cells are extremely low (Fig. IV.6). In this case, a distinct regulation mechanism could be active exclusively for this condition but, to our knowledge, there is no description in yeast of a regulation system involving feedback inhibition by substrate.

Importantly, the significant intracellular glycerol levels reached in *gpd1gpd2* mutant strain grown under salt stress with glycerol (Fig. IV.6/B) point to the activity of a transport mechanism for glycerol. Consistently, in this strain under these conditions, the glycerol uptake observed, which was attributed to Gup2p (see Chapter III), was significantly high and the osmosensitivity compared to

medium with NaCl without glycerol supplementation was lower (see Chapter II). These results strongly support the activity of a transport system for glycerol in which Gup2p is directly involved, being somehow induced by salt stress.

The osmotic tolerance observed in *gpd1gpd2* without exogenously added glycerol cannot be explained exclusively by intracellular trehalose accumulation (Fig. IV.8/B). Therefore, the question of salt stress adaptation in this strain, or more generally, when yeast cells do not synthesise glycerol, remains to be resolved. The pattern of trehalose accumulation is quite different in the strains tested. Intracellular trehalose is maximal in salt stress without external glycerol in W303-1A, while, in *gpd1gpd2*, this value is maximal in salt stress with external glycerol (Fig. IV.8). Blomberg (2000) proposed an adaptation model of yeast cells to osmotic stress involving the activation of ATP-consuming futile cycles through trehalose and glycerol turnover, avoiding the so-called substrate-accelerated cell death (Teusink *et al.,* 1998). Teusink and co-workers (1998), by theoretical considerations, proposed that when cells arrest growth facing stress conditions, the auto-stimulating design of glycolysis, in which ATP formed activates the initial steps of this pathway, would promote accumulation of sugar phosphates, phosphate depletion, and death. Consistently, the lower intracellular levels of trehalose under salt stress with external glycerol (Fig. IV.8/A) suggest lower need for trehalose turnover. On the other hand, in *gpd1gpd2* mutant, trehalose intracellular concentrations are lower than in W303-1A (Fig. IV.8), which is unexpected because the only futile cycle available to consume ATP is by trehalose turnover. Presumably, other physiological mechanisms are acting in this mutant. Marked differences of transcription expression profile induced by salt shock of *gpd1gpd2* strain and W303-1A were determined by microarrays (Yale and Bohnert, 2001). The most up-regulated genes in *gpd1gpd2* strain are genes related to retrotransposons, functionally related to mitochondria, and involved in pheromone response pathways. The way this pattern of transcript induction contributes to osmotic shock adaptation and glycerol synthesis and intracellular accumulation is still not understood.

Interestingly, in the glycerol anabolic pathway, two isoenzymes participate in each metabolic step, encoded by two highly homologous genes. Moreover, the two isoforms are differently regulated and acting in different physiologic mechanisms. *GPD1* and *GPP2*/*HOR2* are mainly involved in osmotic stress response while *GPD2* and *GPP1*/*RHR2* are mainly involved in redox balance (Ansell *et al.,* 1997; Påhlman *et al.,* 2001a). From this study, evidence collected support a difference in regulation between *GUP1* and *GUP2*. Strictly in terms of transcriptional activity in steady-state growth, expression of *GUP2* seems to be more dependent to salt stress than *GUP1*, although the strain in which transcripts of *GUP2* were more abundant is a mutant one unable to synthesise glycerol (Fig. IV.5). These transcript levels are in agreement with physiological data of high glycerol uptake activity under the same conditions. This points to a possible role in salt stress response when cells do not synthesise glycerol as the redox balance is maintained via the electron transport chain in mitochondria. On the other hand, physiological evidence point to *GUP1* as a gene significantly involved in salt stress response, due to slower growth under salt stress with external glycerol observed in the *gup1* mutant (Holst *et al*., 2000). Nonetheless, this phenotype is not concomitant with an activity of glycerol uptake, being, as stated before, probably involved additional roles of Gup1p to cause this phenotype. So, glycerol uptake phenotype revealed by *gup1* mutant strain is only linked to glycerol assimilation under derepression conditions while growth

phenotype is linked to both glycerol assimilation and glycerol-mediated recovery from salt stress in glucose-based medium. Transcript analysis of *GUP1* only points to a possible involvement in fermentative and respiratory metabolisms. For *GUP2*, the sole physiologic evidence points to salt stress response, while transcript analysis although supporting the same function, suggests a possible involvement in fermentative metabolism. As stated above, transcriptional activities do not coincide with those from experiments of osmotic shock using microarrays. Hence, *GUP1* is apparently more involved in osmotic shock than survival and growth under osmotic stress and *GUP2* is apparently more involved in osmotic stress response than in osmotic shock. Differential expression of isoenzymes was observed for several metabolic pathways by genomic-wide expression approaches (Gasch *et al.,* 2000; Causton *et al.,* 2001). Gasch and co-workers (2000) proposed that the reasons for this could be related to different biochemical function, substrate specificity, and physical location. Different biochemical functions for *GUP1* and *GUP2* are likely to occur taking in consideration the different phenotypes observed in different growth conditions, while for substrate specificity, the Km's measured were identical (see Chapter III). Different locations for Gup1p and Gup2p should not be overlooked since localisation studies in Gup1p did not exclude definitely localisations in endoplasmic reticulum and mitochondrial membrane (Holst *et al.,* 2000), which is consistent with localisation predictions, based on primary structure. A localisation in endoplasmic reticulum for Gup2p, besides the most probable plasma membrane localisation, is possible as well (see Chapter III).

Although actively participating in salt stress response, *FPS1* transcription is not regulated by salt stress and is not affected by carbon source like glucose and glycerol (Tamás *et al.,* 1999). This constitutes a resemblance to *GUP1* and *GUP2*, taking in consideration that these genes do not display expression profiles typical of genes known to be salt regulated. In the control of glycerol transport via Fps1p, Tamás and co-workers (1999) demonstrated that the N-terminal hydrophilic extension plays a central role: the deletion of this region causes constitutive transport of glycerol. In this work, regulation of expression of *GUP1* and *GUP2* was demonstrated to be at translational and/or post-translational level. Further investigation is needed to address this question however, the phenotype observed of slower growth and lack of glycerol-mediated recovery from salt stress in *gup1* mutant without detection of glycerol uptake, points to permanent production of the active protein. Therefore, a post-translational control of Gup1p activity seems to be more likely. For *GUP2*, the only phenotype observed was glycerol uptake in glucose-grown cells under salt stress in a mutant strain unable to synthesise glycerol. Nevertheless, transcripts are detected in every growth conditions, similarly to the case of *GUP1*, which might indicate a post-translational regulation as well.

From this work, evidence was found suggesting translational or post-translational regulation of transcriptional activity of *GUP1* and *GUP2* in steady-state growth in ethanol and glucose as carbon sources. The participation in salt stress response in cells adapted and growing under this condition was found to be absent, except for *GUP2* in the *gpd1gpd2* mutant strain. Despite the indications of involvement of *GUP1* in salt shock response (Causton *et al.,* 2001; Yale and Bohnert, 2001), no evidence was found in this work supporting an induction of transcriptional activity in steady-state growth under salt stress. The opposite was found for *GUP2*: absence of transcript levels induction by salt shock and induction by salt stress with concomitant impairment of glycerol

synthesis. In terms of transcriptional activity, these data suggest differential expression of *GUP1* and *GUP2*, although involved in the same physiological mechanism: osmotic adaptation. Nevertheless, these results do not fit the transport measurements made either in wild type or in *gup1* and *gup2* mutant strains, which suggest these genes, in particular *GUP1*, to be connected to respiratory metabolism. Further analysis on promoter sequences of both genes complemented with promoter activity assays by reporter genes will contribute to explain these results. In addition, heterologous expression of *GUP1* and *GUP2* in suitable biological models will contribute to the definitely knowledge of Gup1p and Gup2p functions and will provide the possibility of over-expression of the native proteins, enabling the use of specific antibodies for cellular localisation studies.

Re-assessment of glycerol passive transport in *Saccharomyces cerevisiae*: Fps1p is the mediator of the major part of glycerol passive diffusion

Introduction

The gene *FPS1* was cloned by suppression of the growth defect on fermentable carbon sources of the *fdp1* mutant (Van Aelst *et al.,* 1991). The *TPS1* gene allelic to *fdp1* is involved in trehalose synthesis (Bell *et al.,* 1992) and presumably in the control of sugar influx to glycolysis. These phenotypes, among several others, were suggested by the observation that *fdp1* mutants exhibit low trehalose 6-phosphate synthase (EC 2.4.1.15) activity (Charlab *et al.,* 1985) and, consequently, very low levels of trehalose. When glucose is added to derepressed *fdp1* mutant cells, lethality is caused by extremely high glycolytic flux leading to rapid accumulation of sugar phosphates with depletion of ATP and inorganic phosphate. The limiting step would be the reaction catalysed by glyceraldehyde 3-phosphate dehydrogenase (EC 1.2.1.12), which requires inorganic phosphate as substrate. Van Aelst and co-workers (1991) observed that the presence of additional copies of *FPS1* caused only transient increase of sugar phosphate levels under the same conditions, which is not, presumably, high enough to arrest growth. Luyten and co-workers (1995) explained this effect based on the observation that overexpression of the *GPD1* gene, encoding the key enzyme in glycerol synthesis (glycerol 3-phosphate dehydrogenase; EC 1.1.1.8), caused the same suppression as the *FPS1* gene did. These authors concluded that the increased synthesis of glycerol consumes the sugar phosphates of the upper part of glycolysis and restores the levels of inorganic phosphate. However, the remarkable finding that suppression by *GPD1* was only achieved in the *tps1* mutant and not in the *tps1fps1* mutant suggested that *FPS1* is involved in glycerol metabolism. In addition, the resemblance of sequences of Fps1p with channel proteins of the MIP family of channel proteins, further suggested that the involvement in glycerol metabolism could be at plasma membrane level, presumably, in glycerol efflux (Luyten *et al.,* 1995).

The major intrinsic protein (MIP) family of channel proteins is constituted by membrane proteins of organisms from the five kingdoms (Park and Saier, 1996) functionally involved in cell osmotic balance. A classification based on the permeability of solutes was proposed (Froger *et al.,* 2001), creating the subgroups of aquaporins, specific for water; glycerol facilitators, specific for glycerol or small uncharged molecules; and aquaglyceroporins, permeable to both water and glycerol. Nevertheless, sequence alignment data and data from functional studies suggest that members of this family of proteins have closely related physiological functions (Park and Saier, 1996). The function of osmotic balance maintenance is in fact consistent with the type of transported molecules of the members of the MIP family. The transport of water is considered the first adaptation to osmotic stress because when different water activities occur between the cytoplasm and the external medium, water moves to compensate and balance the water activities (Bonhivers *et al.,* 1998). On the other hand, glycerol is the compatible solute employed to decrease intracellular water activity in yeasts, allowing to compensate external hyperosmolarity and to avoid desiccation caused by the initial response of water efflux.

Features shared among members of the MIP family include the existence of six predicted transmembrane domains and several conserved residues (Reizer *et al.,* 1993). The two asparagine-proline-alanine (NPA) sequences are the most

remarkable of these motifs being, one localised in the loop between transmembrane domains 2 and 3, and the other between helices 5 and 6. The so-called "hourglass" model of MIP channel proteins was proposed by Jung and co-workers (1994) based in biochemical and biophysical data. In this model, loops containing NPA motifs constitute hemipores that overlap midway between the transmembrane domains, creating a pore, presumably, for the transported molecules. Other features are shared only by members of the same functional category and have been proposed to be involved in substrate specificity. These include conserved residues at specific positions and the amino acid content and length of predicted loop regions (Froger *et al.,* 1998). The oligomerization state of MIP channel proteins was proposed to be specific as well, being the organization of glycerol facilitators as monomers and the one of aquaporins as tetramers (Lagrée *et al.,* 1998). These authors demonstrated the conversion of the oligomerization state of an aquaporin to a monomer characteristic of glycerol facilitators, by substitution of a serine residue, conserved at this position in aquaporins, by an aspartic acid residue, which is conserved in glycerol facilitators at the same position. In a subsequent work, Lagrée and co-workers (1999) reported an alteration of the transported molecule, from water to glycerol, by the substitution of two amino acids in the insect aquaporin AQPcic.

Bill and co-workers (2001) emphasized the notion that distinct structural features can be the basis for different physiological function and specificity of substrate. Fps1p has been considered as an atypical member of the glycerol facilitator subgroup of the MIP family due to the long amino- and carboxyl-terminal extensions and to the lack of the characteristic NPA motifs, being Asn-Pro-Ser (NPS) in the loop between transmembrane domains 2 and 3 and Asn-Leu-Ala (NLA) in the loop between transmembrane domains 5 and 6 (Hohmann *et al.,* 2000). According to Bill and co-workers (2001), the differences of the length of both terminal extensions could be attributed to the regulation by osmotic stress of Fps1p, which is totally absent in the more typical glycerol facilitator of *Escherichia coli* GlpF. In addition, these authors reported remarkable differences in pore flexibility between Fps1p and GlpF caused, partly, by the lack of NPA motifs in Fps1p. The influence of osmotic stress upon Fps1p causes closure of the channel, in which the N-terminal extension was demonstrated to be involved (Tamás *et al.,* 1999; Tamás *et al*., 2003). The existence of a flexible pore is consistent with such mechanism of conformational change involved in regulation of Fps1p driven by osmotic stress. Therefore, the differences observed between Fps1p and other glycerol facilitators, particularly GlpF, may be explained by these features, together with the involvement in glycerol efflux and the capacity to transport arsenite (III) and antimonite (III) (Sanders *et al.,* 1997; Wysocki *et al.,* 2001). These differences could justify a different transport mechanism for Fps1p and GlpF as well. It is known that GlpF functions merely as a channel for passive diffusion of glycerol (Heller *et al*., 1980) and the transport through Fps1p was reported to be by facilitated diffusion (Luyten *et al*., 1995; Sutherland *et al.,* 1997). These authors attributed to Fps1p the saturable glycerol uptake kinetics detected in cells grown in media with glucose as carbon and energy source. According to the same authors, this type of kinetics was absent in the *fps1* mutant, being detectable only passive diffusion with a much lower K_d than in wild type cells. A partial suppression of this phenotype was observed with GlpF. The Fps1p transport mechanism was considered a facilitated diffusion because the K_m had a much higher value than the one of active transport. Furthermore, it was not concentrative and it was not sensitive to the action of the ionophore CCCP. However, the unusual saturable nature of a channel-mediated

transport has raised doubts on the transport mechanism. In addition, the example of *E. coli* GlpF activity which has been shown to be tightly connected to glycerol kinase activity (Voegele *et al.*, 1993) and the results from Holst and co-workers (2000) who described an influence of this enzyme activity in active glycerol transport measurements further challenge the accepted transport mechanism for Fps1p. In this work, a possible interference by Gut1p on glycerol uptake experiments employed to determine the activity of Fps1p, is studied. Furthermore, the mechanism for glycerol uptake is re-evaluated and discussed, taking in consideration the concept of glycerol liposolubility.

Materials and Methods

Yeast strains, media and growth conditions

Cells of strains listed in Table V.I were grown in rich medium [1% (w/v) yeast extract and 2% (w/v) peptone] containing 2% (w/v) glucose (YPD) as carbon and energy source or 2% (w/v) ethanol (YPE) as carbon and energy source. Growth was performed at 30ºC in Erlenmeyer flasks with medium/air relation of 1/1 and agitation at 160 r.p.m. Growth was followed by spectrofotometry at 600 nm.

Table V.I. Yeast strains used in this work.

Strain	Genotype	Origin/reference
W303-1A	*MAT***a** *leu2-3, 112 ura3-1 trp1-1 his3-11, 15 ade2-1 can1-100*	Thomas and Rothstein, 1989
---	Isogenic to W303-1A except *fps1*::*LEU2*	Van Aelst *et al.,* 1991
Cly1	Isogenic to W303-1A except *gut1*Δ	Holst *et al.*, 2000
BHY40	Isogenic to W303-1A except *gut2*Δ	Holst *et al.*, 2000

*Portuguese Yeast Culture Collection (Universidade Nova of Lisbon, Portugal)

Glycerol transmembrane transport studies and glycerol kinase assays

Sample preparation, preparation of radioactive glycerol solutions, initial uptake rates of radiolabelled glycerol and radiolabelled glycerol intracellular accumulation were performed according to the procedures described in Chapter III. Similarly, experimental determination of glycerol kinase activity was done as described in Chapter III.

Quantification of culture medium solutes by high performance liquid chromatography (HPLC)

Cells from 1.5 ml aliquots of cultures were removed by centrifugation (7400x*g*, 5 min, 4ºC). Proteins were precipitated with 2% (v/v) perchloric acid and incubation in ice for 30 min. Precipitated proteins were separated by centrifugation at 10,000x*g*, 4ºC for 10 min and supernatant was collected and stored at -20ºC. For injection on HPLC column, samples were diluted with equal amount of internal standard (10 g/l arabinose). Results are mean values of, at least, three independent experiments.

Northern analysis

For RNA isolation, cells were harvested by centrifugation in mid-exponential growth phase (OD_{600} = 0.4-0.5) and in late exponential to stationary phase (OD_{600} = 1.1-1.2), and washed twice with cold deionised water. RNA was isolated and analysed as described before (see in Chapter IV Materials and Methods). All solutions were prepared with DEPC-treated water and when non-disposable labware was used, RNases were heat-inactivated. Total RNA (30 µg) was separated by size in a denaturing agarose gel [1,2% (w/v)] electrophoresis in MOPS running buffer (40 mM MOPS, pH 7.0; 10 mM sodium acetate; 1 mM EDTA). Gels were prepared with MOPS running buffer, including 0.66 M formaldehyde as denaturing agent. RNA samples were mixed with MOPS running buffer, 2.2 M formaldehyde, and 50% (v/v) formamide, and denatured at 55ºC for 15 min. Immediately after heat denaturation, samples were transferred to ice and mixed with formaldehyde loading buffer 6x [1 mM EDTA; 0.25% (w/v) bromophenol blue; 50% (v/v) glycerol] to 1x final concentration. Samples were loaded on the gel and electric field strength of 5 V/cm was applied until bromophenol blue reached 1-2 cm from the edge of the gel. After electrophoresis, gels were washed three times with ultra-pure water for 10 min to remove formaldehyde and, subsequently, washed twice with SSC 10x (dilution from SSC 20x stock solution: 3 M NaCl, 0.3 M trisodium citrate, pH 7.0 adjusted with HCl) for 5 min.

RNA was immobilised by capillary transfer to a nylon membrane GeneScreen Plus (NEN-Life Sciences Products), according to standard procedures (Ausubel *et al.,* 1996; Farrell Jr, 1996) with SSC 10x as transfer buffer. Crosslinking of RNA in the nylon membrane was performed by UV irradiation. Probes for *FPS1* and internal standard, rRNA 18S, were obtained by PCR, using genomic DNA as template for *FPS1* and cDNA for internal standard. Forward primer for *FPS1* probe was 3'CCTACAGTCTTGCCCTCCAC5' and reverse 3'AACATTCCCGCAACACTTTC5'. Forward primer for internal standard was 3'AGGAATTGACGGAAGGGCAC5' and reverse 3'GGACATCTAAGGGCATCACA5'. PCR products were purified with diatomaceous earth (High Pure PCR Product Purification Kit – Roche) and labelled with alkaline phosphatase with Alkphos Direct labelling Reagents (Amersham Pharmacia Biotech). Membranes were prehybridised with AlkPhos Direct hybridisation buffer (Amersham Pharmacia Biotech) at 55ºC for 15 min, and labelled probes were added to the buffer to a final concentration of 10 ng/ml. Hybridisations were made at the same temperature for 16-18 hours. Post hybridisation washes included pre-heated

primary wash buffer [2 M urea; 0.1% (w/v) SDS; 50 mM Na_3PO_4; 150 mM NaCl; 1 mM $MgCl_2$; 0.2% (w/v) blocking reagent] to 55ºC and incubation twice at this temperature for 10 min. Subsequent washes were made with secondary wash buffer (50 mM tris base; 100 mM NaCl; 2 mM $MgCl_2$; pH 10.0), twice, at room temperature for 5 min. For signal generation and detection, CDP-Star™ Detection Reagent (Amersham Pharmacia Biotech) was used at 40 µl/cm^2, for 5 min. Wrapped membranes were placed in a film cassette with blots facing the autoradiography film (Hyperfilm™ ECL), left to expose for 1 hour and developed. Images were digitalized and densitometric analysis was performed using NIH Image 1.60 software. For each sample, the ratio between the signal of the target gene and the signal generated by the internal standard was used to overcome variability between samples such as RNA quality and RNA quantification errors. Results are mean values of, at least, three independent experiments.

Results

In previous works, low affinity glycerol transport has been reported as absent in *fps1* mutants, which was considered as an evidence for a facilitated diffusion mechanism for Fps1p (Luyten *et al.,* 1995; Sutherland *et al.,* 1997). Glycerol uptake experiments were performed in order to confirm the detection of a facilitated diffusion activity in *fps1* mutants and to check a possible influence of Gut1p activity on facilitated diffusion determination. Glycerol uptake was determined in cells collected at exponential and diauxic shift growth phases of glucose batch cultures and at the exponential growth phase of ethanol batch cultures (Fig. V.1). Glucose-grown cells of both strains presented first-order kinetics characteristic of passive diffusion when collected during exponential growth phase and a saturable component of low affinity, when cells were collected during the diauxic shift (Fig. V.1). In intracellular glycerol accumulation experiments performed in diauxic shift cells grown on glucose, no substantial in/out accumulation ratios were reached for these strains (Fig. V.1/inserts). Interestingly, accumulation ratios are higher (around 3) in W303-1A than in the *fps1* mutant (around 1) being, W303-1A intracellular radioactivity accumulation linear over time, which indicates incorporation of the substrate by metabolism and not the intracellular accumulation against gradient of free glycerol. On the other hand, in cells grown in ethanol and harvested at exponential growth phase, considerable in/out ratios were attained. Similarly, a striking difference between these two strains was detected with higher accumulation ratio by *fps1*.

Several data of glycerol uptake obtained are intriguing due to the fact that they are not fully interpretable in the context of a transport by facilitated diffusion that has been attributed to Fps1p. The constitutive nature of *FPS1* expression as observed by others (Luyten *et al.,* 1995; Sutherland *et al.,* 1997; Tamás *et al.,* 1999) would allow the detection of a saturation kinetics for glycerol uptake in cells grown in glucose and harvested at exponential growth phase. However, this was not observed in this work, except for cells harvested at diauxic shift growth phase (Fig. V.1/A). So, the facilitated diffusion mechanism attributed to Fps1p was questioned and the possibility that the saturation kinetics observed could be due to an artefact was investigated.

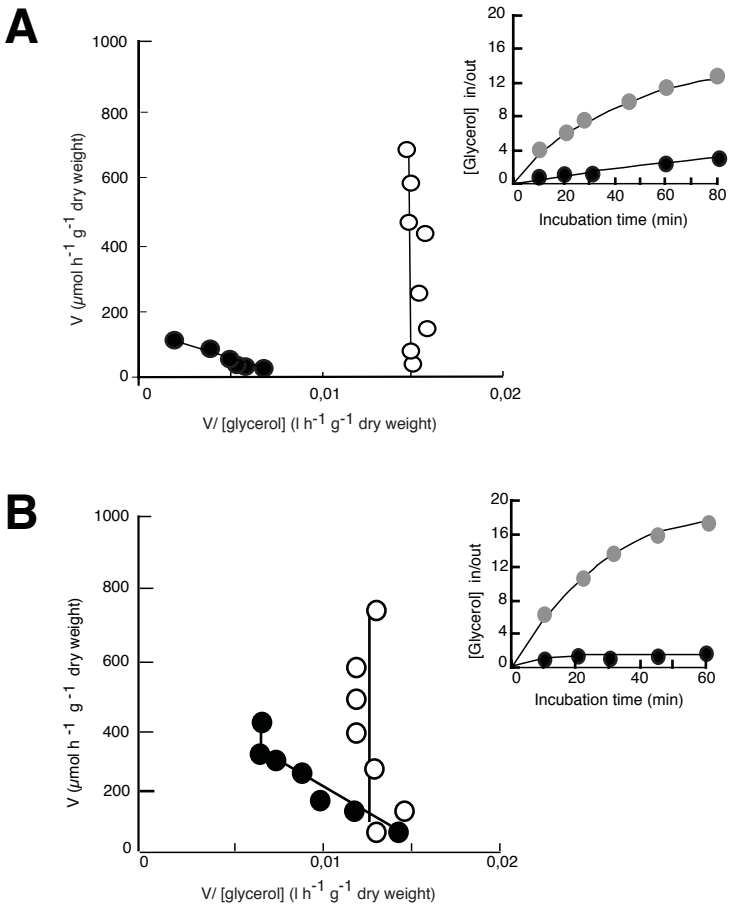

Figure V.1. Eadie-Hofstee plots of initial uptake rates of [^{14}C]glycerol in cells grown in YPD and collected in exponential growth phase (❍) and in the diauxic shift (●) of *S. cerevisiae* strains W303-1A (A) and *fps1* (B). In inserts are represented the in/out accumulation ratios of [^{14}C]glycerol in cells harvested in diauxic shift growth phase of YPD batch culture (❍) and in exponential growth phase of YPE batch culture (●).

With this evidence pointing to an artefact as the source of the saturable transport detected in diauxic shift, glycerol kinase activity would be the most probable cause of interference on glycerol uptake experiments the same way as reported by Holst and co-workers (2000). Therefore, in order to study this possibility, glycerol kinase activity was measured in cells harvested at different phases in batch cultures of YPD, including early-exponential, late-exponential, early-diauxic shift, and late-diauxic shift growth phases and were tested for glycerol kinase activity (Fig. V.2). To correlate enzymatic activities with repression/derepression conditions, external glucose and ethanol were quantified at each time point. Partial derepression of glycerol kinase is detected in the *fps1* mutant and in a lesser extent in W303-1A when glucose is less than 10g/l in the medium. A remarkable difference is the higher activity of glycerol kinase at any growth phase in the *fps1* mutant when compared with the parental strain W303-1A. Glycerol kinase activities obtained with this mutant were rather high, reaching, at late-diauxic shift growth phase, approximately half the value obtained in derepressed cells by growth with ethanol as carbon and energy source (68 mU.mg^{-1} protein). Hence, the remarkable parallelism between

detection of glycerol kinase activity and saturation kinetics of glycerol uptake, suggested that the kinase could actually be the cause of the saturation kinetics.

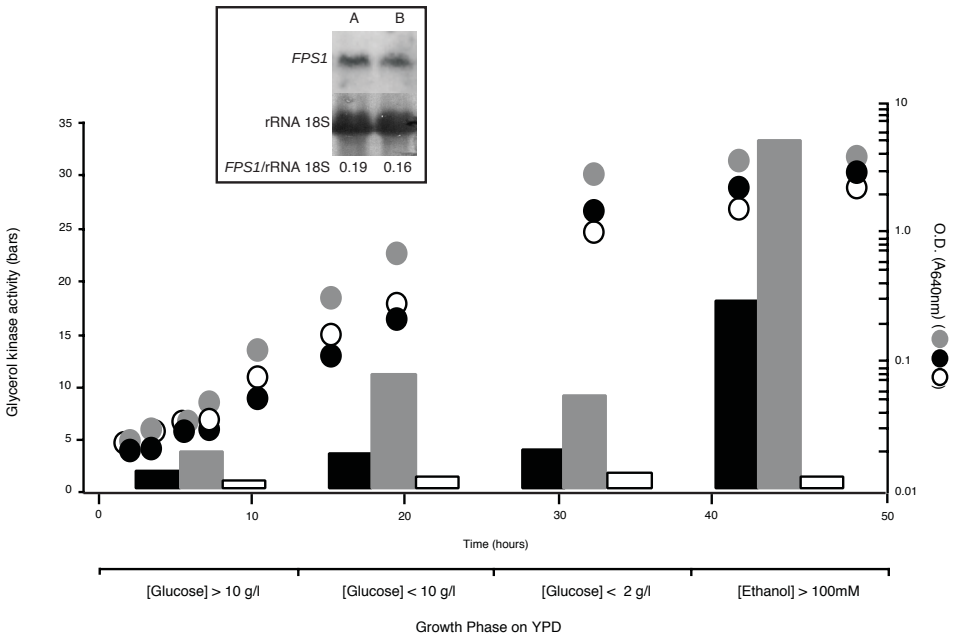

Figure V.2. Variation of glycerol kinase activity along growth on YPD medium of *S. cerevisiae* strains W303-1A (black symbols and bars), *fps1* mutant (grey symbols and bars) and *gut1* mutant (white symbols and bars). The variation of extracellular glucose and ethanol along growth is also shown below the graphic. Insert: relative quantification of *FPS1* transcripts by Northern analysis in cells harvested at mid-exponential growth phase (A) and at the diauxic shift (B).

To determine if the saturable kinetics detected in diauxic shift cells could be caused by an induction of *FPS1* transcription, we analysed expression of *FPS1* in W303-1A cells grown in rich medium with glucose as carbon and energy source and harvested in mid-exponential and in diauxic shift growth phases. The intensity of blots did not change between mid-exponential and diauxic shift cells (Fig V.2/insert), in accordance to works with different strains under the same growth conditions, using Northern analysis (Tadi *et al.,* 1999) and microarrays technology (DeRisi *et al.,* 1997). Therefore, these results clearly indicate that transcripts of *FPS1* are invariant when cells grow on glucose up to diauxic shift phase (OD_{600} ≈ 1.2) and that the saturable kinetics characteristically detected at this growth stage is unlikely to be the activity of Fps1p. Nevertheless, the possibility of post-translational control of *FPS1* expression cannot excluded by these results.

The influence of catabolism on experimental determinations of glycerol uptake was further tested in mutants affected in glycerol kinase encoded by *GUT1* and in mitochondrial glycerol 3-phosphate dehydrogenase (EC 1.1.99.5) encoded by *GUT2*. Glycerol uptake and intracellular accumulation experiments were performed in *gut1* and *gut2* strains in cells grown on glucose and harvested at diauxic shift phase and cells grown on ethanol and harvested at exponential growth phase (Fig. V.3). As expected, glycerol uptake is clearly different in these strains being, detected only passive diffusion in the *gut1* strain and a further component with saturation kinetics in *gut2* (Fig. V.3/A). These results strongly

suggest that glycerol kinase interferes in glycerol uptake experiments creating a transport measurement artefact like had been observed with active transport (Holst *et al.*, 2000). In addition, in/out glycerol accumulation ratios do not exceed equilibrium in *gut1*, without any observable effect by CCCP, while in *gut2*, as expected, some intracellular accumulation is detected and an effect is detected by CCCP (Fig. V.3/A-inserts). However, this effect was not total, presumably, because some imported glycerol was phosphorylated by glycerol kinase. When ethanol-grown cells were used (Fig. V.3/B), the characteristic bi-phasic kinetics was detected with the components of first order kinetics and a second one corresponding to the active uptake system (Lages and Lucas, 1997; Holst *et al.,* 2000). The accumulation ratios, as expected, were higher for both strains when compared with glucose-grown cells (Fig. V.3/B-insert). In the *gut1* strain efflux by "cold" glycerol was until equilibrium is reached and accumulation prevention by CCCP was total, which is consistent with the absence of catabolism of glycerol. On the other hand, in *gut2* "cold" glycerol promoted an efflux until an in/out ratio of 2, which, despite the fact that CCCP prevents completely intracellular glycerol accumulation, indicates that some glycerol is being converted in glycerol 3-phosphate.

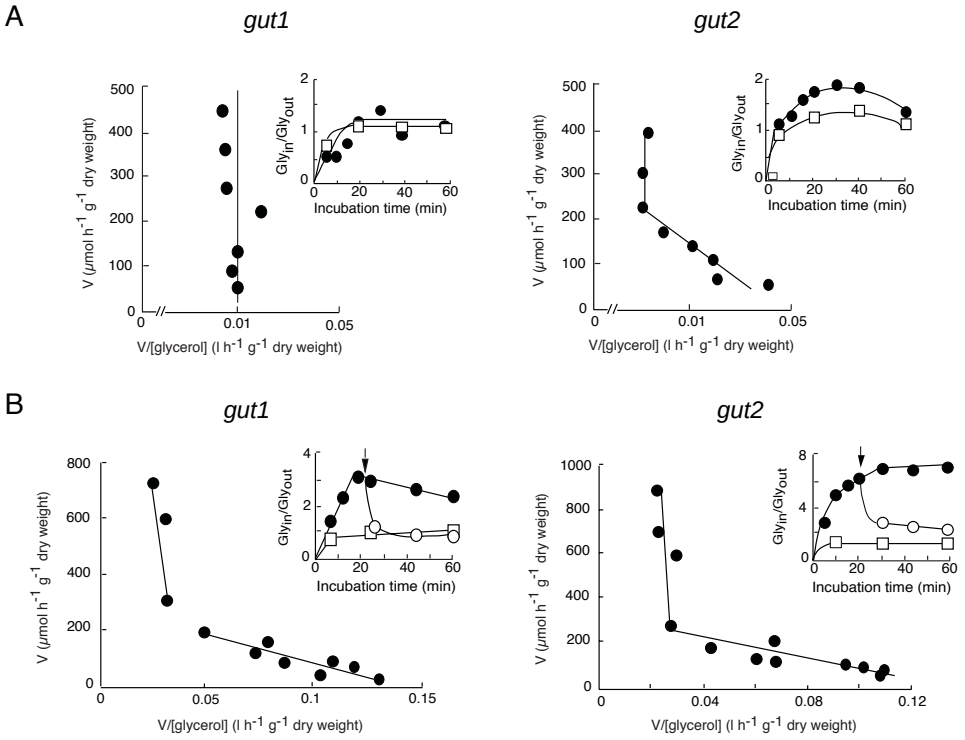

Figure V.3. Eadie-Hofstee plots of [^{14}C]glycerol initial uptake rates in cells *S. cerevisiae* mutant strains *gut1* and *gut2* grown on YPD and harvested at the diauxic shift (A) and grown on YPE and harvested at mid-exponential growth phase (B). Inserts: in/out accumulation ratios of [^{14}C]glycerol in the absence (●) and in the presence of the ionophore CCCP (❑). In [^{14}C]glycerol accumulation experiments, efflux of [^{14}C]glycerol was obtained with non-radiolabelled ("cold") glycerol (❍).

Discussion

In this work, evidence is presented suggesting a mechanism of passive diffusion for glycerol uptake through Fps1p and that the saturation kinetics detected in glucose-grown cells is an artefact caused by partial derepression of glycerol kinase encoded by *GUT1*. Facilitated diffusion of glycerol through this MIP channel protein has been considered the most plausible mechanism (Luyten *et al.,* 1995; Sutherland *et al.,* 1997). Glycerol uptake with saturation kinetics in glucose-grown cells was detected exclusively at the diauxic shift phase (Fig. V.1/A), which is not consistent with a constitutively expressed *FPS1*, assuming that its protein product is responsible for facilitated diffusion of glycerol. Moreover, in salt-shocked cells a saturable glycerol uptake was observed (Oliveira *et al*., 2003), which is not in accordance with the regulation mechanism by closure upon osmotic shock attributed to Fps1p (Tamás *et al.,* 1999). Finally, *fps1* mutant strain presented saturable glycerol uptake under the same conditions as the parental strain W303-1A (Fig. V.1/B). This uptake was only absent in mutant strain *gut1* (Fig. V.3/A), lacking the enzyme catalysing the first catabolic step of glycerol: glycerol kinase.

An effect of salt shock on glycerol uptake has been reported only when high concentrations of substrate are used (Lages, 2000). On the other hand, in the range of substrate concentrations used to determine saturation kinetics (low concentration), no effect has been reported, suggesting that only channel-mediated passive transport is sensitive to this stress. These observations together with the known characteristic low permeability of *S. cerevisiae* plasma membrane to glycerol, at least when compared to *Candida utilis* (Gancedo *et al.,* 1968), suggest that the retention component contributing to global glycerol intracellular accumulation lies in the plasma membrane and acts through a mechanism of passive diffusion. A rapid alteration on yeast cells under osmotic shock is cell shrinkage (Albertyn *et al.,* 1994b; Lages and Lucas, 1997; Lages *et al.,* 1999) and a consequence of this is lipid packing at the plasma membrane. Therefore, the plasma membrane could be this regulating element allowing diffusion of glycerol and decreasing permeability by lipid packing upon osmotic shock. On the other hand, a channel protein would fit this model as well due to changes in lipid packing that could induce conformational changes on the protein (de Marañón *et al.,* 2001).

Glycerol uptake experiments with *fps1* mutant strain (Fig. V.1/B) seem to contradict results previously published by Sutherland and co-workers (1997) in particular the detection of uptake with saturation kinetics. However, these authors performed these experiments in mid-exponential growth phase cells, being detected, only passive diffusion as in this work. Here, saturation kinetics is detected in diauxic shift cells, which constitutes a marked physiological difference. In W303-1A strain, however, results do not match for the same growth phase (Fig. V.1/A). One possibility could be the low V_{max} calculated from these experiments (Sutherland *et al*., 1997) that can cause difficulties in data analysis. The noticeable glycerol uptake with saturation kinetics in the *fps1* mutant is not in accordance with the mediated diffusion model for Fps1p. The GlpF channel of *E. coli*, a typical member of the functional subgroup of glycerol facilitators belonging to the MIP family of channel proteins, uptake of glycerol was reported to be by passive diffusion (Heller *et al.,* 1980). Therefore, according to the observations of Lages (2000) of salt shock sensitivity by passive diffusion

rather than mediated transport, the possibility of Fps1p to function as a channel was considered. Intracellular glycerol accumulation in diauxic shift cells, as expected, did not exceed equilibrium, although W303-1A strain exhibited in/out ratios above 1 in a linear fashion over time (Fig. V.1/inserts). The difference observed in both strains can be explained with *FPS1* deletion. The permeability to glycerol in the *fps1* strain is lower enough to hamper an incorporation of radiolabelled substrate, at least, to levels observed in the parental strain. This strongly suggests that the Fps1p-dependent transport contributes in high proportion to overall plasma membrane permeability to glycerol. In ethanol-grown cells, the higher levels of intracellular accumulation in the *fps1* mutant are consistent with absence of efflux (Luyten *et al.,* 1995; Tamás *et al.,* 1999) in glycerol-loaded cells through the activity of the glycerol/H^+ symporter (Lages and Lucas, 1997; Holst *et al.,* 2000).

Partial derepression of Gut1p was demonstrated in diauxic shift when glucose concentration is below 10 g/l (Fig. V.2). In experiments with constructions of fused *GUT1* promoter with *lacZ* reporter gene, significant repression was still observed in 2 g/l glucose (Grauslund *et al.,* 1999). Nevertheless, higher promoter activity was observed under these conditions compared to 20 g/l glucose, which is consistent with increased glycerol kinase activity observed in this work. Beginning of derepression of *GUT1*, by relative quantification of transcripts using microarrays, was observed in batch cultures when glucose concentration was around 2 g/l (DeRisi *et al*., 1997; Gasch *et al*., 2000). In addition, in similar experiments to the ones presented here, evidence of partial derepression of *GUT1* in early-diauxic shift cells was obtained (Lages, 2000). Derepression of *GUT1* is more efficient when glycerol concentration is significant (20 g/l) than ethanol, lactate, or acetate in similar concentrations (Sprague and Cronan, 1977; Grauslund *et al.,* 1999). So, different levels of expression have been observed for *GUT1*, which is in accordance with partial derepression when glucose concentration is very low. The only factor affecting *GUT1* promoter activity seems to be glucose because blends of 1% glucose with 1.5% glycerol caused the same effect on promoter activity than just 2% glucose (Grauslund *et al.,* 1999). However, in this work, partial derepression was detected when glucose concentration was below 1% with simultaneous presence of ethanol produced by fermentation. The lack of catabolite inactivation of glycerol kinase by glucose reported by Sprague and Cronan (1977) is concordant with the possibility of low levels of *GUT1* expression when glucose is still present in the medium with simultaneous presence of increasing amounts of ethanol. This way, at the diauxic shift growth phase, significant levels of glycerol kinase activity can be detected, which can contribute to the observed saturation kinetics in experiments of glycerol uptake.

As pointed out above, glycerol kinase activity interferes with glycerol uptake experimental determinations (Fig. V.3). This interference was observed for both glucose-grown and ethanol-grown cells and affected uptake kinetics and in/out accumulation ratios. These observations are in accordance with the lack of influence of salt stress upon the mediated component of experimentally determined glycerol uptake (Lages, 2000), assuming that this mediated component is actually the activity of the glycerol kinase. Remarkably, in these experiments with salt-shocked cells, the mediated component attributed to Fps1p has been detected which is not consistent with the regulation of Fps1p by salt stress, causing closure of the channel (Tamás *et al*., 1999). Therefore, the model of passive diffusion for Fps1p-mediated glycerol transport is fully consistent with

these reports. The experimental determination of this uptake is affected by the activity of glycerol kinase when growth conditions allow, at least, partial derepression as has also been reported in glycerol active uptake studies (Holst *et al*., 2000).

The mechanism of passive diffusion proposed here for the activity of glycerol transport by Fps1p, is in conflict with the idea of high lipid solubility of glycerol. In fact, if glycerol can cross the plasma membrane freely, the function of Fps1p would be redundant. However, glycerol is mentioned to be insoluble in oils as in other lipid-soluble compounds and miscible in water and alcohol (O'Neil *et al.,* 2001). Accordingly, oil/octanol partition coefficient (logP) of glycerol (-4.15) is quite different to a lipid-miscible compound like ethanol (-1.33) and is even lower to sugars like glucose (-1.96) and fructose (-1.77) (Leo *et al.*, 1971). Lower diffusion constants in the *fps1* mutant than the correspondent parental strain were observed in cells grown on ethanol (Lages, 2000) and on glucose (Sutherland *et al.,* 1997). In addition, the growth defect of *fps1* under anaerobic conditions is consistent with an inability to export synthesised glycerol for redox regulation, suggesting low efficiency of glycerol diffusion through the plasma membrane (Tamás *et al.,* 1999). So, these data point to Fps1p, acting as a channel, as the main contributor to the plasma membrane permeability to glycerol. Nevertheless, simple diffusion through the plasma membrane should not be disregarded since the plasma membrane is not a simple model composed by a lipid bilayer. Proteins embedded in the membrane could change locally physical properties of the membrane and, hence, make possible simple diffusion of glycerol or other uncharged small compounds.

Several effects caused by *FPS1* deletion and over-expression suggest additional roles for this gene. Deletion mutants exhibit a phenotype of fusion defect, suggesting osmotic control for the degradation and fusion of membranes during mating (Phillips and Herskowitz, 1997). Regulation of plasma membrane lipid composition, and hence control of its permeability, was suggested by different content in glycolipids and phospholipids presented by *fps1* mutant and its parental strain (Sutherland *et al.,* 1997). A function of carrier to other substrates than glycerol was proposed by Wysocki and co-workers (2001) such as arsenite and antimonite. Other evidence suggests involvement in additional functions, apart from the function of channel, directly related with glycerol metabolism. So, increased intracellular and extracellular glycerol content was reported upon *FPS1* over-expression (Luyten *et al.,* 1995). Still upon over-expression of *FPS1*, V_{max} of the H^+/glycerol symporter and K_d were approximately duplicated (Lages, 2000). In *FPS1* deletion mutants, higher growth rates than the parental strain were measured in glucose, glycerol, and ethanol (Lages, 2000; Fig. V.2) and significantly higher activity of glycerol kinase was detected in glucose-grown cells from early-exponential to late-diauxic shift growth phases (Fig. V.2). While the effects of over-expression on K_d and V_{max} of active uptake and the deletion on growth rates are difficult to explain, the effect of the deletion on glycerol kinase seems likely due to the fact that catabolism remains, in this case, as the only pathway to decrease intracellular levels of glycerol. This is in accordance with the observation that *fps1* mutants do not exhibit growth phenotype changes when cultured with glycerol as carbon and energy source, which points to a negligible role of influx by opposition to efflux (Tamás *et al.,* 1999). Therefore, *FPS1* function would be strictly related to control of intracellular levels of glycerol through efflux and regulation of metabolism, while a connection to glycerol uptake for growth seems unlikely. The

maintenance of correct intracellular levels of glycerol is very important in growth under anaerobiosis and osmotic down-shock, which correspond to environmental conditions of growth defect of *fps1* mutants (Tamás *et al.,* 1999).

ChapterVI

General conclusions

At the beginning of this work, transmembrane transport of glycerol in *Saccharomyces cerevisiae* cells has been considered to be through three mechanisms: active uptake (Lages and Lucas, 1997), facilitated diffusion (Sutherland *et al*., 1997) and passive diffusion (Lages and Lucas, 1997; Sutherland *et al*., 1997). The model that has been suggested to explain glycerol uptake kinetics included three different conditions of metabolic regulation. In derepressed cells by growth on non-fermentable carbon sources like ethanol and glycerol, in both defined synthetic and rich complex media, a non-saturable first order kinetics together with a component of second order are detected, which were attributed to simple diffusion and active transport respectively. The mechanism of active transport has been reported to be of symport with protons as suggested by extracellular alkalinisation of cell suspensions by addition of glycerol. The *FPS1* gene known to be involved in glycerol uptake (Luyten *et al*., 1995), was demonstrated to be unrelated with the active transport since this H^+/glycerol symport is still detected in *fps1* mutant cells (Sutherland *et al*., 1997).

In repressed cells, harvested at the exponential growth phase of cultures with glucose as carbon and energy source, in both synthetic and complex media, are detected simple diffusion and a second component with low affinity saturable kinetics. Fps1p was considered to mediate the second component since it was reported to be absent in *fps1* mutant cells. The reported involvement of Fps1p in glycerol efflux in order to regulate its intracellular concentration (Luyten *et al*., 1995; Tamás *et al*., 1999) would allow then to balance the high glycerol synthesis rate in fermenting cells to regulate redox potential. In the specific case of diauxic shift, cells have exhausted glucose and are reprogramming gene expression to respiratory metabolism. Despite the fact that repression by glucose is absent, active uptake was not detected. The kinetics of glycerol uptake is similar to exponential growing cells on glucose (Sutherland *et al*., 1997). However, results concerning the so-called Fps1p-mediated low affinity facilitated diffusion have not been consensual, since the saturable uptake component was not detected according to different reports (Lages, 2000; this work, Chapter V). This difference has been suggested to occur due to differences in growth rates of W303-1A and *fps1* strains that would prevent synchronicity of growth phase when cells are harvested for experiments (Oliveira *et al*., 2003).

Several evidences point to different roles of the gene *FPS1*, according to phenotypes revealed by the *fps1* mutant strain. Nevertheless, the role as glycerol channel has been strengthened with growing evidence pointing to involvement in glycerol influx and efflux (Sutherland *et al*., 1997; Tamás *et al*., 1999; Oliveira *et al*., 2003). One of these roles for *FPS1*, suggested by differences in glycolipid and phospholipid composition of the plasma membrane in the wild type and the *fps1* mutant strain, is an involvement in lipid composition of the plasma membrane. This could be the reason for the marked difference of the passive diffusion coefficient observed between these strains cultivated in rich complex medium with glucose as carbon and energy source (Sutherland *et al*., 1997).

Several lines of research were needed to improve the model of the glycerol transmembrane transport: identification of the gene encoding the active transport system, clarification of the transport mechanism through the Fps1p channel and the influence of *FPS1* on passive diffusion through the plasma membrane. In this work, cloning of the gene encoding the glycerol active transport system was tried (Chapter II) and the mechanism of transport through the Fps1p channel was determined together with new insights on the glycerol passive diffusion (Chapter

V). After cloning the genes involved in active glycerol uptake, physiological characterisation of the respective mutant strains was done in order to establish this function to the cloned genes (Chapter III). Subsequently, expression analysis was performed to understand the physiological role of each one (Chapter IV).

The strategy for cloning the gene encoding the glycerol active transport system involved the participation of this transport system in osmotic stress response through the contribution to glycerol retention for higher intracellular concentration. When overexpressed in a multicopy plasmid from a *S. cerevisiae* genomic library, the gene would contribute to complementation of the osmosensitivity of the *gpd1gpd2* mutant strain. Several reasons were indicated to explain the unsuccessful cloning including the low V_{max} of the system that would not be enough to cause a physiological effect under the conditions of the screening. However, the genes *GUP1* and *GUP2* were cloned with a similar strategy but through a negative selection (Holst *et al.*, 2000), in oposition to the screening of this work. Therefore, a negative selection in the screening seems to be more appropriate in particular for genes that confer weak phenotypes. In fact, in addition to low V_{max} of glycerol uptake, it was still possible to detect growth in plate assays to test glycerol utilisation as carbon and energy source and salt stress sensitivity in the *gup1* mutant and absence of phenotype in the *gup2* mutant (Holst *et al.*, 2000).

Glycerol uptake experiments and intracellular accumulation assays in mutants affected in *GUP1*, *GUP2* and in several genes encoding enzymes involved in glycerol metabolism allowed to obtain evidences supporting the involvement of *GUP1* and *GUP2* in glycerol active uptake. However, glycerol uptake is only completely abolished in mutant strains affected simultaneously in *GUP1* and *GUT1*, which indicates an interference of the first step of glycerol catabolism, catalysed by glycerol kinase encoded by *GUT1*, in experimental determinations of glycerol uptake. The only effect observed caused by *GUP2* deletion was in cells with a *gpd1gpd2* genetic background grown on glucose in the presence of NaCl and glycerol. Under these conditions a surprisingly high V_{max} value in glycerol uptake was observed, which was still considerably high in a strain with further deletion of *GUP1*. Only when *gpd1*, *gup1* and *gup2* mutations are present in the yeast strain, the glycerol uptake is abolished. The involvement of two highly homologous genes with different regulation in the same metabolic step of glycerol metabolism is found in the reduction of dihydroxyacetone phosphate reduction (*GPD1* and *GPD2*) and in the dephosphorylation of glycerol 3-phosphate (*GPP1* and *GPP2*). In the case of glycerol active uptake, evidence support that *GUP1* is involved in growth on glycerol as a sole carbon and energy source while *GUP2* is apparently involved in salt stress response in cells grown on glucose.

Transcription of *GUP1* and *GUP2* is constitutive being detected relatively constant levels of mRNA for both genes in cells grown on glucose and ethanol. In addition, transcriptional activity is not significantly changed in cells of rich complex media cultures with fermentative metabolism, respiratory metabolism and in the diauxic shift. An exception was the low level of *GUP1* mRNA in the diauxic shift, which might indicate an increased activity in exponential growing cells with fermentative or respiratory metabolism. *GUP1* and *GUP2* transcription was not significantly changed with salt stress as well. Taking in consideration physiological data concerning glycerol transmembrane transport and expression analysis of *GUP1* and *GUP2*, the regulation of glycerol uptake seems to be at

translational and/or post-translational levels. Interestingly, regulation of the activity of the Fps1p glycerol channel occurs at post-translational level through a mechanism of opening and closing the pore with the involvement of the N-terminal domain of the protein (Tamás *et al.*, 1999; Tamás *et al*., 2003).

The accepted concept of liposolubility of glycerol is not fully concordant with the low diffusion constants determined (Sutherland *et al.*, 1997; Chapter III) and with the involvement of Fps1p in intracellular glycerol regulation. The suggested influence of *FPS1* in plasma membrane lipid composition (Sutherland *et al*., 1997) is a plausible explanation but the relation between the lipid composition changes and the decreased plasma membrane permeability remains to be demonstrated. However, as stated in Chapter V, glycerol is not a liposoluble compound. Therefore, passive diffusion is very low and glycerol transmembrane movements are confined to plasma membrane carriers like Fps1p, Gup1p and Gup2p. The regulation role of Fps1p is then to control intracellular levels by retention of glycerol inside the cell and by allowance of diffusion to abolish concentration gradients.

The mechanism of transport of Fps1p was re-investigated due to inconsistent data of lack of saturable uptake kinetics in glucose-grown cells harvested at exponential growth phase and the mechanism of facilitated diffusion. The saturable uptake kinetics is only detectable in the diauxic shift. An interference of the first metabolic step of glycerol phosphorylation by glycerol kinase was observed in uptake experiments in mutants affected in the *GUT1* gene encoding this enzyme. A similar interference was also demonstrated in active uptake experiments under derepressing conditions in ethanol-grown cells (Chapter III). In the case of the diauxic shift, however, this interference is due to partial derepression of *GUT1* when external glucose is consumed as demonstrated in this work. The saturable kinetics of glycerol uptake is abolished in the *gut1* mutant, suggesting that the Fps1p-mediated glycerol uptake is characterised by a first order kinetics. Therefore, in this work the model of simple diffusion through Fps1p is proposed in oposition to the previous model of facilitated diffusion.

As has been demonstrated in Chapter III, *GUP1* and *GUP2* genes are clearly involved in glycerol active uptake. However, conclusive evidence indicating that *GUP1* and *GUP2* encode the active transport systems has not been obtained. This involvement might be explained by the activity of Gup1p and Gup2p as regulatory proteins or as plasma membrane sensors. The evidences pointing to Gup1p and Gup2p as transport systems are, in our opinion, the phenotypes revealed by the mutant strains affected in *GUP1*, *GUP2* and *GUT1* in what concerns glycerol uptake. The lack of glycerol active uptake in the *gut1gpd1gup1* and *gut1gup1gup2* in ethanol-grown cells and the abolishment of glycerol uptake only when *GUP1* and *GUP2* are deleted in a *gpd1gpd2* genetic background in salt-stressed cells (Chapter III) are strong evidences supporting this role. Other phenotypes of the *gup1* mutant might be explained as indirect effects of glycerol uptake impairment. These phenotypes include impairment of growth on glycerol as sole carbon and energy source and the salt stress sensitivity phenotype (Holst *et al*., 2000). On the other hand, evidences have been obtained that point to different roles, without excluding conclusively the glycerol uptake role. These evidences include new phenotypes attributed to the *gup1* mutation like temperature sensitivity and increased triglyceride synthesis concomitant with decrease phospholipid synthesis (Oelkers *et al*., 2000). More evidences come from sequence similarities of *GUP1* and *GUP2* with *ARE1* and *ARE2* encoding

sterol *O*-acyltransferases and the inclusion in the MBOAT (for membrane-bound *O*-acyl transferases) superfamily (Hofmann, 2000; Oelkers *et al.*, 2000). The involvement in lipid metabolism suggested by these phenotypes is remarkable since the role in glycerol uptake could be physiologically related taking in consideration that glycerol is a precursor of lipid biosynthesis. Hence, for instance, a role in synthesis of phospholipids for the plasma membrane, involving esterification of glycerol, or glycerol derivatives, as *O*-acyltransferases is consistent with known data concerning *GUP1* and *GUP2*.

With data presented in this work it is possible to improve the model of glycerol transport in *S. cerevisiae*. In derepressed cells, Gup1p mediates glycerol active uptake and the derepression of *GUT1* allows glycerol entry, presumably by passive diffusion as well, with immediate phosphorylation, which contributes to saturable uptake kinetics determined experimentally under these conditions. In repressed cells at the exponential or diauxic shift growth phases, active uptake is inhibited by a translational or post-translational mechanism, passive diffusion is mediated by Fps1p and, in a lesser extent, passive diffusion occurs through the plasma membrane. In the diauxic shift by exhaustion of glucose, saturable uptake kinetics is determined experimentally due to the partial derepression of *GUT1* with subsequent phosphorylation of incoming glycerol and creation of a gradient favourable to influx. Under salt stress, Gup2p, as well as Gup1p, are active and contribute to intracellular concentration of glycerol by taking up external glycerol when glycerol biosynthesis is deficient or impaired.

Despite the fact that the involvement of *GUP1* and *GUP2* in glycerol active uptake is unequivocally demonstrated, the demonstration that Gup1p and Gup2p are the transport systems responsible for active uptake is still not fully accomplished. Therefore, future work should provide a clear answer to this issue. Biological systems known to lack this type of uptake should be used to assay heterologous expression of *GUP1* and *GUP2*. A good candidate for such system is the fission yeast *Schizosaccharomyces pombe,* for which only passive diffusion has been determined (Lages *et al.*, 1999). However, this yeast has a *GUP1* homologue gene (Chapter III), which suggest that active uptake might occur probably under conditions not tested. An alternative system of plasma membrane vesicles has been used successfully used for functional reconstitution of transport systems (Gerós *et al.*, 1996; Marobbio *et al.*, 2002). In addition, plasma membrane vesicles provide good systems for uptake kinetic studies due to absence of interfering internal metabolism. Given the low V_{max} of glycerol uptake (Chapter III) and the low level of transcription of *GUP1* and *GUP2* (Chapter IV), a suitable strategy to obtain a considerable amount of purified Gup1p and Gup2p is to isolate these proteins by affinity chromatography with columns containing a specific antibody attached to the stationary phase. Specific antibodies can also be useful to demonstrate cellular localisation of Gup1p and Gup2p. The widely used approach of protein fusions with the green fluorescent protein from the jellyfish *Aequorea victoria* can be suitable as well. However, due to the low level of expression of *GUP1* and *GUP2*, detection can become difficult. So, specific antibodies labelled with fluorescent probes can provide clear demonstration of localisation of the glycerol transport systems.

Regulation of expression of *GUP1* and *GUP2* should be further investigated as well. As discussed in the General Introduction (Chapter I), glycerol is involved in several regulatory mechanisms. These include redox and inorganic phosphate regulation, which are involved in glycolysis flux control and stress response such

as osmotic, oxidative and high temperature. Expression analysis of these genes and physiological data suggest some involvement in osmotic stress response and in utilisation of glycerol as carbon and energy source. Regulatory and sensor functions have not been excluded from analysis of data of this work. Therefore, promoter analysis might provide important indications on the function and physiological purposes of *GUP1* and *GUP2*. This analysis should include identification of sequences specific for transcription factors and consensus regions with other promoters of genes of glycerol and lipid metabolisms. Subsequent expression analysis in mutant strains with increasingly longer partial deletions of the promoter in order to exclude some of these specific sequences would indicate the most important upstream regulatory elements. By identification of the signal transduction pathways that interact with these transcription factors, it will be possible to classify *GUP1* and *GUP2* in terms of cellular function. Finally, combination of mutations in the regulatory pathways and in *GUP1* and *GUP2* can uncover new features of these genes that, eventually, can be useful for metabolic improvement of engeneered strains for industrial application.

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Symbols and abbreviations

A - Absorbance
ATP - Adenosine 5'-triphosphate
BLAST - Basic local alignment search tool
cAMP - Cyclic adenosine 5'-monophosphate
CCCP - Carbonyl cyanide m-chlorophenylhydrazone
CDP - Cytidine 5'-diphosphate
Ci - Curie
CoA - Coenzyme A
DEPC - Diethylpyrocarbonate
dpm - Disintegrations per minute
dNTP - Deoxynucleotide triphosphates
DTT - Dithiotreitol
EDTA - Ethylenediamine tetraacetic acid
FAD - Flavin adenine dinucleotide
HPLC - High-perfomance liquid chromatography
K_d – Diffusion constant
K_m - Michaelis constant
MES - 2-(N-Morpholino)ethanesulfonic acid
MIP family - Major intrinsic protein family
MOPS - 3-(*N*-Morpholino)propanesulfonic acid
NAD^+ - β-nicotinamide adenine dinucleotide
NADH - β-Nicotinamide adenine dinucleotide, reduced form
ORF - Open reading frame
PEG – Polyethylene glycol
PCR - polymerase chain reaction
ΔpH - Transmembrane H^+ concentration gradient
pmf - Proton-motive force
$\Delta\Psi$ - Electrical transmembrane potential difference
RT-PCR - Reverse transcribed polymerase chain reaction
RT-PCR - Reverse transcriptase polymerase chain reaction
SDS - Sodium dodecyl sulfate
Tris - Tris[hydroxymethyl]aminomethane
triton X-100 - t-Octylphenoxypolyethoxyethanol
UV - Ultraviolet
V_{max} - Maximum velocity